The Nematode *Caenorhabditis elegans* as an Alternative Model for Bacterial Infection

Thesis

The Nematode *Caenorhabditis elegans* as an Alternative Model for Bacterial Infection

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Submitted for the Degree of Doctor of Philosophy

Bacterial Pathogenesis

Sponsored by the Defence Science Technology Laboratory (dstl), Chemical and Biological Sciences, Porton Down.

2004

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Chapter: 2.

Abstract

The usefulness of the nematode *Caenorhabditis elegans* as a model of bacterial infection has been assessed. Initially a plate-based mortality assay was established using *Pseudomonas aeruginosa*. A novel method for assessing bacterial virulence towards *C. elegans* in liquid media was also developed. These assays were used to investigate the virulence of strains from the *Burkholderia cepacia* complex, *Burkholderia pseudomallei* and *Burkholderia thailandensis*. The results from the plate-based mortality assay mirrored the virulence of *B. pseudomallei* and *B. thailandensis* in mammals. However the results also showed that this method was not suitable for the investigation of the *B. cepacia* complex. Assays carried out in liquid media were not a robust measure of bacterial virulence for any of the *Burkholderia* strains tested.

The *C. elegans* model was also used to investigate virulence mechanisms of *B. pseudomallei*. *B. pseudomallei* can adhere to and invade a variety of mammalian cell types. Transmission electron microscopy was used to investigate the interactions of *B. pseudomallei* with the cells of the *C. elegans* gut. No evidence of adherent bacterial cells or invasion of the tissues surrounding the gut was seen. A panel of *B. pseudomallei* transposon mutants was screened in *C. elegans*. A single transposon mutant was identified that was attenuated in nematodes and was subsequently also shown to be attenuated in BALB/c mice. The interrupted gene encoded a putative aldehyde dehydrogenase. This protein has not been previously shown to play a role in the virulence of *B. pseudomallei*. The nematode model was also used to screen a *B. pseudomallei* genomic cosmid library in *E. coli* for clones with increased virulence. However the data indicated that this method could not be utilised for the identification of *B. pseudomallei* virulence genes without further development.

Declaration of content:

All electron microscopy was performed by Mr. A. B. Dowset of the Health Protection Agency (HPA) Salisbury, Wiltshire, UK.

A library of transposon mutants of the bacterium *Burkholderia pseudomallei* was used in this study. This was created and supplied by Dr. T. P. Atkins of dstl, CBS Porton Down, Salisbury, Wiltshire. UK.

A library of bacterial clones carrying cosmid vectors containing genomic DNA from the bacterium *B. pseudomallei* was used in this study. This was created and supplied by Dr. C. Winstanley of the Department of medical microbiology and GU medicine, University of Liverpool, Liverpool, UK.

Sections of the work presented in chapter: 3 of this thesis, regarding the feeding inhibition assay were published in FEMS microbiology letters in 2002 and has the following citation; Smith, M.P., Laws, T.R., Atkins, T.P., Oyston, P.C.F., De Pomerai, D.I., Titball, R.W. (2002) A liquid based method for the assessment of bacterial virulence using the nematode *Caenorhabditis elegans*. FEMS microbiology letters. 210 (2) PP. 181-185.

None of the material submitted in this thesis has been previously submitted for a degree or any other qualification at the Open University or any other institution.

Acknowledgements:

I wish to thank all of those people that have given me support and advice while writing this thesis including my mentor Tim Atkins and colleagues Tom Laws, Joanne Thwaite, Matali Sarkar-Tyson, Steve Mitchell, Barry Dowset for the advice he gave regarding electron microscopy and finally Clare Lynex for her discussions in molecular biology. Special thanks goes to my PhD supervisors Prof. R. W. Titball and Dr. P. C. F. Oyston for their hard work and guidance through the time it has taken to produce this thesis. Finally I would like to thank my wife Star as without her understanding and support this work would have never been finished. I would also like to thank Theresa Stiernagle as some of the nematode strains in this work were provided by the CGC, which is funded by the NIH National Centre for Research Resources (NCRR). This work was funded by the Defence Science Technology Laboratories (Dstl) Porton Down.

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List of Abbreviations.

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
cDNA	Cloned deoxyribonucleic acid
M	Moles
mM	Milli-moles
AMP	Adenosine monophosphate
ADP	Adenosine diphophate
NADP	Nicotinamide adenosine diphosphate
CD	Cluster of differentiation
IL	Interlukin
TNF-α	Tumour necrosis factor-alpha
PAF	Platelet aggregating factor
VPI	*Vibrio cholerae* pathogenicity island
bp	Base pairs
Kb	Kilo-base pairs
Mb	Mega-base pairs
U	Units of activity
ml	Milli-litres
µl	Micro-litre
g	Grams
mg	Milli-grams
µg	Micro-grams

kD	Kilo-daltons
dH_20	Distilled water
μF	Micro-faridays
kV	Kilo-volts
Ω	Ohms
MA	Milli-amps

Chapter 1: Introduction

1.1. Bacterial pathogenicity and infectious disease of the mammalian host.

Only a small proportion of the bacterial species associated with humans give rise to the biological changes that are interpreted as disease by clinicians. When these bacteria are actively growing within the host, the host is said to be experiencing an infection (Prescott 1996). Infectious disease may then result when part or all of the host body becomes incapable of carrying out normal functions due to the presence of an organism or its products. Any bacterium that causes disease is referred to as a pathogen and its ability to cause disease is termed pathogenicity (Prescott 1996).

To cause disease a pathogen must be capable of carrying out five obligatory steps. That is to enter the host, attach to host cells or surfaces with invasion of these cells in some cases, multiply within the host, avoid host defence mechanisms and finally cause damage to the host (Prescott 1996). Many of these steps are mediated by virulence factors (Casadevall.A and Pirofski.L 1999). A range of virulence factors are used by bacterial pathogens to cause an infectious disease. As it is not possible to provide an exhaustive description of every virulence factor used in each stage of infection by every bacterial pathogen of man, only examples of the best characterised of these and their role in each stage of infection are discussed below.

1.1.1. Entry.

The first feature in the initiation of infectious disease is the transmission and entry of the pathogen into the host. The mammalian body is covered by relatively impermeable skin and this along with resident skin flora present a considerable barrier to infection. However the skin does not cover all body surfaces and is replaced by mucous membranes at the conjunctiva of the eye, in the alimentary tract, in the urogenital tract and in the respiratory tract. These offer routes of infection to pathogenic organisms as the penetration of these surfaces is more easily accomplished than of intact skin (Mims 1997).

Pathogens may enter host tissues after direct contact with other infected individuals, as is the case with many sexually transmitted bacterial diseases such as gonorrhea caused by *Neisseria gonorrhoeae* (Prescott, Harley *et al.* 1996). Infectious organisms may also be shed into the environment during infection. This allows possible contamination of soil, food and water sources. Some environmental bacteria have the potential to cause disease in man. Thus pathogenic organisms can be ingested or inhaled from the environment or from other infected individuals, to cause infection of either the respiratory or alimentary tract. Additionally pathogens may also enter the body in an opportunistic fashion through breaks in the skin from wounding events or from pre-existing conditions that result in the disruption of the normal barriers to infection such as immunodefficiency (Mims 1997).

Some pathogenic organisms are also transmitted via vectors such as biting insects (Mims 1997). This allows pathogens that are not normally capable of penetrating unbroken skin to enter a host. This is particularly exemplified by the transmission of *Yersinia pestis*, the etiological agent of bubonic plague. *Y. pestis* persists in certain rodent populations in some

parts of the world and is transmitted primarily by the rat flea *Xenopsylla cheopis* (Hinnebusch 1996). After being ingested in a blood meal from an infected host, the bacteria multiply in the flea gut and form a mass that can occlude the proventriculus, a spined chamber located between the flea oesophagus and midgut, which prevents the flea from feeding. During persistent attempts to feed on a new host the flea regurgitates infected blood containing *Y. pestis* into the bite site, transmitting the organism to the mammalian host (Hinnebusch 1996).

Thus it is evident that some bacterial species have mechanisms that allow them to be transmitted from host to host, through dissemination into the environment or through infection of a vector that in turn allows infection of the definitive host. This however is only the first stage in the initiation of any infection and an outcome that results in disease is also dependent on the subsequent stages described below.

1.1.2. Attachment and invasion.

The adherence of a pathogen to host mucosal or cell surfaces is often pivotal in the establishment of infection. Two common mechanisms are employed by bacterial pathogens to attach themselves to host cells. This is through the use of fimbrae or pili and afimbrial adhesins. These allow a colonising organism to resist the physical removal by host clearance mechanisms such as peristalsis in the gut and hydrodynamic forces in the urinary tract. The abolishment of the ability of a bacterial pathogen to attach and colonise a specific host tissue, through adhesin-mediated processes, is often sufficient to cause the attenuation of the organism indicating the importance of these structures in virulence (Hultgren 1993; Klemm 2000). Bacterial adhesins can also be responsible for the tropism for particular tissues seen in bacterial diseases such as that caused by *N. gonorrhoeae* in the urinary tract or diarrhoea-causing *Escherichia coli* in the gastro-intestinal tract of humans (Nassif 1999).

The best understood mechanism of adherence to host cells is through attachment mediated by fimbral adhesins also termed pili (Salyers 1994). Most bacterial adhesins are organised as organelles on the bacterial cell surface with lengths of approximately 1μm. Some are thin fibres with diameters of about 2 nm. Others are more rigid structures with diameters of 5-10 nm (Klemm 2000). The bulk of the organelle generally consists of a complex of a single protein subunit called pilin, which is arranged, in a helical array to form a long cylindrical structure. This allows the formation of a long flexible structure that emanates outward from the bacterial surface. The role of these structures is to establish initial contact between the bacterial surface and the outer surface of the host cell. The tip of the fimbriae mostly consists of a specialised structure, containing proteins distinct from the structural

pilin subunits. Often it is these tip structures that mediate binding to host cell surface ligands such as carbohydrate residues and glycolipids (Salyers 1994). In some cases the structural protein itself can also act as the specific adhesin as is the case with the CFA-1 fimbriae of entero–pathogenic *E. coli* (EPEC).

The biosynthesis and assembly of fimbriae is a complex process. The secretion of pilin and other proteins involved in fimbriae biogenesis is usually mediated through the normal protein secretion mechanisms of the organism. However in some instances a fimbriae-specific system secretion system is used. There are two fundamentally different mechanisms used for the secretion of pili to their final site on the bacterial surface. This is represented by the autotransporter pathway and the other more complex chaperone-assisted surface display mechanism. The AIDA-1 adhesin of certain EPEC strains is responsible for the diffuse adherence to the intestinal mucosa (Klemm 2000). It belongs the autotransporter protein family that encompasses many other virulence factors and is characteristic in that the protein itself contains the required information to reach the bacterial cell surface and lacks a cleavable signal peptide as in some other secretion mechanisms.

However, in most Gram-negative species many adhesins are displayed on the bacterial surface by chaperone-assisted transport, an illustrative example being type-1 fimbriae of *E. coli* (Klemm 2000). In general, chaperones aid in the transport of proteins to the outer membrane. The adhesive tip structure is assembled and the shaft of the fimbriae created. This occurs through the sequential addition of structural subunits to the proximal part of the structure, in doing so pushing the assembled portions outward from the bacterial membrane. Finally a periplasmic protein halts the extrusion process (Salyers 1994).

A prime example of the function of fimbrial adhesins as virulence factors is shown by the PapG adhesin of P fimbriae present on strains of uropathogenic *E. coli*. This is an essential virulence factor in the establishment of pyelonephritis by allowing specific binding to receptors found in the globoseries of the human kidney (Klemm 2000). Similarly the FimH adhesin of type-1 fimbriae has also been shown to contribute to the virulence of uropathogenic *E. coli* strains in the urinary tract (Bahrani-Mougeot 2002). Deletion of these pathogenicity determinants causes a corresponding decrease in virulence.

Afimbral or non-pilus adhesins are proteins that although important for the adherence of bacteria to host surfaces do not assemble themselves into fimbriae-like structures. These cell surface proteins often mediate the tighter binding of bacteria to the host cell following the initial binding via fimbriae (Salyers 1994).

An example of the importance of afimbrial adhesins in pathogenicity is shown by non-capsulated strains of *Hemophillus influenzae*. To cause disease *H. influenzae* must first colonise the respiratory tract. As with other Gram-negative bacteria this organism expresses adhesive fimbriae that are involved in colonisation. However, organisms that lack these fimbriae also remain capable of binding to eukaryotic cells suggesting the presence of afimbrial adhesins. It was subsequently found that *H. influenzae* expresses two such proteins designated HMW1 and HMW2. Both of these proteins show homology with filamentous hemagglutanin, an afimbrial surface protein of *Bordetella pertusis* . They also appear to play a critical role in adhesion *in vivo* to human epithelial cells (Hultgren 1993).

After initial attachment some pathogens may invade host cells. This occurs through normal phagocytic processes or the induction of actin rearrangements at the cell surface that mediate engulfment in normally non-phagocytic cells. This is induced by a class of

bacterial surface proteins collectively called invasins (Salyers 1994). After ingestion by host phagocytic cells some pathogens can also escape into the cytoplasm of the cell through disruption of the phagosome. Once in the cytoplasm some bacterial pathogens continue to exploit actin to allow motility. Here condensation of actin at one end of the organism propels the bacterium through the host cells cytoplasm and into adjacent cells. This phenomenon is particularly seen in *Listeria* and *Shigella* species which have become the paradigm of intracellular invasion and motility (Salyers 1994).

The adherence to and possible invasion of host cells represents the first stage in the establishment of a bacterial infection, where host-pathogen interactions that occur on a cellular level, determine the out come of that infection. If successful in colonising host tissues a pathogen must then multiply to allow sufficient numbers of organisms to overwhelm any immune response mounted by the host.

1.1.3. Multiplication within the host.

To grow and multiply within a host a pathogen must first find or produce an environment that meets its particular requirement for nutrients, pH, temperature and redox potential (Prescott 1996). Some pathogens invade and grow within host cells such as the *Listeria* species already mentioned. This includes professionally phagocytic cells such as macrophages. Invasion allows access to an abundance of nutrients and provides protection from host defence mechanisms and some antibiotics (Salyers 1994). *Listeria* species have mechanisms that allow them to survive and replicate intracellularly. This includes the production of Listeriolysin O, a hemolysin used to rupture the phagocytic vacuole and facilitate the escape of the organism into the cytoplasm. Additionally *Listeria* species produce the enzymes catalase and superoxide dismutase. These aid in protection from the bactericidal oxidative burst within the phagolysosome of machrophages by scavenging oxidative free radicals (Salyers 1994).

Other intracellular pathogens utilise other survival strategies that allow persistance within host phagocytic cells. This includes the interference of phagosome and lysosome fusion, as seen in *Mycobacterium tuberculosis* infection of human macrophages and monocytes. This interruption inhibits the release of the bactericidal contents of the lyosome into the phagosome allowing survival of the organism (Malik 2000). Some pathogens also actively resist the change in internal environment within the phagolysosome. This is shown by *Salmonella enterica* serovar Enterica, which is able to survive the highly acidic conditions within the phagolysosome after lysosomal fusion. This occurs through a process called the acid tolerance response and allows survival of the organism and replication within macrophages (Clements 2001).

Extracellular bacterial pathogens often produce virulence factors that also aid in the spread of the organism from the site of initial infection. *Staphylococcus aureus* is the causative agent of a variety of both community-acquired and nosocomial infections. These range from superficial skin infections to life threatening conditions such as pneumonia (Sifri 2003). The ability of *S. aureus* to cause a wide spectrum of disease is attributed to its ability to produce a broad array of virulence factors. These include hyaluronidase, which allows the hydolysis of hyaluronic acid in the basement membranes of eukaryotic cells rendering intracellular spaces amenable to passage by the pathogen (Prescott 1996). *S. aureus* may also produce fibrinolysin. This mediates the digestion of fibrin clots at infection sites allowing the pathogen to move from clotted areas within wounds into underlying tissues (Prescott 1996) thus contributing to the spread of the organism.

Perhaps the most important attribute of a successful bacterial pathogen that facilitates multiplication with in the host is the ability to acquire iron from the environment. Iron is essential for the multiplication of all bacterial pathogens as it is a component of many enzymes which are required for the biosynthesis of macromolecules such as DNA and energy-generating electron transport systems (Rabsch 1999). However, it has been estimated that *in vivo* the concentration bioavailable Fe^{3+} is 10^{-18} M (Poole 1996). This is several orders of magnitude less than the 10^{-7} M required for bacterial growth (Braun 2002). Bacterial pathogens employ a variety of mechanisms to acquire iron from the environment. However the most common method involves the production of high affinity iron chelators called siderophores (Poole 1996). These bind iron ouside of the cell and transport it through the bacterial outer membrane via specific receptors (Meyer, Neely *et al.* 1996).

Transcription of siderophore biosynthetic genes is tightly regulated by intracellular iron concentration and ferric uptake regulation (Fur) proteins (Ratledge 2000). When the intracellular concentration of iron is sufficient the synthesis of the siderophore and corresponding transport proteins is shut off. This occurs through binding of ferric ions to the proteins Fur and DtxR, which when bound to iron act as transcriptional repressors through binding to promoters containing a Fur binding sequence (Fur box). Positive regulation of siderophore synthesis and iron uptake may also occur in some systems through extracytoplasmic sigma factors (Braun 2002; Crosa 2002). These regulatory mechanisms allow bacteria to activate and repress sets of genes that encode proteins for the assembly and export of siderophores and the specific uptake of the iron-siderophore complexes (Crosa 2002).

After liberation into the external milieu and binding of iron, the uptake of siderophore-iron complexes requires special mechanisms of ferric ion transport. In Gram-negative bacteria, this is achieved through the binding of the iron -carrying compounds to specific outer membrane proteins that function as transporters. After binding the iron complexes are released and transported across the outer membrane into the periplasm where they bind to transporter proteins. These deliver the iron to the ATP binding cassette (ABC) transporters of the cytoplasmic membrane which coveys it to the cytoplasm (Braun 2002). The siderophore component may then be recycled to be used again to acquire further supplies of iron.

The release of siderophore- iron complexes from the binding site and subsequent transport across the outer membrane is an energy consuming process. The bacterial outer membrane contains no energy source due to the lack of any membrane potential. To ameliorate this problem Gram-negative bacteria have developed a system for energising the outer

membrane through the proton motive force of the cytoplasmic membrane (Braun 2002). This is achieved in *E. coli* through the Ton system, which consists of three proteins TonB, ExbB and ExbD. TonB spans the periplasmic space and shuttles between cytoplasmic and outer membrane during energy transduction with ExbB and ExbD responsible for its localisation in the cytoplasmic membrane (Higgs 2002). The proteins further interact with each other and form a complex that responds to proton motive force by binding a proton. This causes a structural change in TonB that converts it to an energised form. This further interacts with the outer membrane transporters causing ferric complexes to disassociate from their binding sites and diffuse through the channel that is simultaneously opened to the cytoplasm (Braun 2002).

Many siderophores are capable of removing iron from host iron binding proteins such as lactoferrin and transferrin in *vitro* and thus may contribute to the ability of many organisms to survive and grow in the host (Meyer, Neely *et al.* 1996). Siderophores are then considered as important virulence factors in many bacterial pathogens, which allow the invading pathogen to grow and multiply in spite of the iron depleted conditions encountered *in vivo*.

1.1.4. Avoidance of host defence mechanisms

A pathogen once within the host body is faced with a variety of non-specific and specific defence mechanisms that it must overcome to be able to effectively produce disease. Thus bacterial pathogens have evolved a number of mechanisms that allow them to circumvent the host immune response and promote persistence within infected tissues, a selection of which are described below.

The disabling of the cells that mediate host antibacterial responses is one method used by pathogens to allow active avoidance of host defences. In many cases this is achieved through the use of type III secretion (TTS) mechanisms. This allows the contact-dependant delivery of effector molecules directly into a target cell's cytoplasm with a variety of effects on the host (Feltman, Schulert *et al.* 2001). Similar secretory pathways have been identified in a variety of animal pathogens including *S. flexneri*, *S. enterica*, EPEC and enterohemorrhagic *E. coli* (EHEC). However, one of the most completely described TTS systems is the Ysc-Yop virulence apparatus of pathogenic *Yersinae* which include *Yersinia enterolitica*, *Y. pestis* and *Yersinia peudoturberculosis*.

The Ysc-Yop TTS of *Yersinia* species consists of a TTS apparatus termed the Ysc injectosome and set of proteins secreted by the system called Yops, which have a variety of detrimental effects on eukaryotic cells and in particular those of the immune system (Cornelis 2002). These are encoded on a 70 kb plasmid that is essential for virulence in animal models of infection (Haller 2000). Four of these Yop proteins exert a negative effect on the cytoskeletal dynamics of host cells. YopH is a phosphotyrosine phosphatase (Haller 2000) that disrupts the adhesion of macrophages and reduces invasin-mediated

engulfment of cells. T and B cells exposed to YopH are impaired in their ability to be activated through their antigen receptors. Thus YopH contributes not only to the evasion of the innate immune response but may also incapacitate the host adaptive immune response. Furthermore YopE, YopT and YopO all exert their effects on the actin cytoskeleton. This leads to morphological changes in host cells, which have been shown to be essential in resistance to macrophage mediated killing (Cornelis 2002). Other identified Yop proteins do not affect the structural components of host cells but serve to modulate the immune response. YopP/YopJ once injected, is able to counteract the normal pro-inflamatory response in a variety of cell types and induces apoptosis specifically in macrophages. As well as the reduction of the release of TNF-α and IL-8 by macrophages it also reduces the presentation of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and E-selectin, reducing the recruitment of neutrophils to the site of infection. This occurs as a result of the inhibition of the activation of NF-κB, a transcription factor central to the onset of inflammatory reactions (Cornelis 2002). Finally the protein YopM also contributes to virulence. It belongs to a family of type III effector molecules that have representatives in *Shigella* and *Salmonella* spp. YopM is delivered into the cytoplasm of target cells where it traffics to the nucleus through the use of a vesicle-associated pathway. However, so far its action in the nucleus has not been elucidated (Cornelis 2002).

Thus TTS systems can be important virulence factors that allow pathogenic organisms to effectively defend against phagocytic cells of the immune system. These systems may also modulate the signalling cascades responsible for induction of immune responses, abrogating their antimicrobial function and allowing survival within the host.

The possession of a TTS system is only one example of a bacterial virulence factor that allows active protection of pathogenic species within a host through the attack of immune

cells. However other mechanisms exist that provide a more passive form of protection through resistance to host defence strategies. Many pathogens synthesise exopolysaccharides (EPS) which in most cases are polymers consisting of repeating units of simple sugars. These may remain attached to the cell through a lipid moiety as in Gram-negative species or appear covalently linked to the outer membrane as seen in Gram-positive organisms. These are referred to as capsular polysaccharides or capsules. However, EPS is also found in an unattached form surrounding the bacterial cell. In these cases the term slime layer or S-layer is used to describe the often loosely fitting structure (Llull 2001).

EPS production is usually associated with an increase in virulence in many pathogens as it may help the organism to avoid host defence mechanisms. The possession of a capsule inhibits activation of the complement cascade and subsequent opsonisation and killing by bactericidal complement components of normal serum. It is also an important determinant in the resistance against phagocytic-mediated killing (Llull 2001). Although often weakly immunogenic capsular polysaccharides may modulate immune functions through influencing the production of cytokines such as the pro-inflammatory TNF, IL-8 and IL-10 (Steinmetz, Nimtz *et al.* 2000). Furthermore EPS can be produced under conditions of environmental stress and play a key role in the formation of biofilms that can confer an increase in antibiotic resistance to the colonising organism. This is of particular concern in the hospital environment where colonisation of medical equipment such as indwelling catheters by pathogenic organisms is a continuous problem (Llull 2001).

Many resistance mechanisms employed by pathogenic bacteria focus on the avoidance of host defence mechanisms through the interference with these processes, as described in the sections above. However some bacterial pathogens may also camouflage their products

from host defence mechanisms through mimicry of host processes or host antigens. In some cases mimicry is achieved through virulence factors that are direct homologues of host proteins. In others, evolution has produced molecules that although not similar at the amino acid level display mimicry at the structural level (Stebbins 2001). This is particularly exemplified by the hyaluronic acid capsule of some *Streptococcus* species. The structure of this extracellular component is identical to components of mammalian connective tissue (Mims 1997). Furthermore the cytoplasmic membrane of *Streptococcus pyogenes* contains antigens similar to those of human cardiac, skeletal, and smooth muscle, heart valve, fibroblasts, and neuronal tissues, resulting in molecular mimicry and a tolerant or suppressed immune response by the host (http://www.bact.wisc.edu/Bact330/lecturespyo).

A similar mechanism for avoidance of host immune responses is through antigenic variation. This allows a rapid change in the antigenic structure of bacterial surface proteins that ensures that any specific immune response raised against these proteins is rendered ineffective. This mechanism is seen in the structural components of pili and other outer membrane proteins from *N. gonorreae.* The pilin subunits *of N. gonorreae* consist of constant, variable and hypervariable regions analogous to immunoglobulin molecules (Mims 1997). Thus spontaneous genetic rearrangements and recombinations occurring within genes encoding pilin proteins ensures that a different antigenic target is constantly presented to the host immune system. This reduces the effectiveness of the immune response induced and allows persistence of the organism within affected tissues.

Avoidance of host immune responses does not always require the possession of specialised cellular structures. Several pathogens exploit areas that are inaccessible to host defence mechanisms. This includes survival within host cells and also cells of the immune response

itself such as macrophages. For example *Shigella* invade mucosal cells of the colonic mucosa through the M-cells of the payer's patches. The organism then ruptures the phagocytic vacuole in which it is internalised and divides rapidly. Lateral movement through the polymerisation of actin allows the infection of other adjacent cells. Hence the bacterium is effectively protected from the host immune response, as exposure to host microbicidal processes is limited (Salyers 1994).

The small selection of virulence factors and mechanisms thus far described allow a pathogen to colonise, grow and persist within an infected host. However to produce disease a pathogen must also be able to cause damage to the host. This involves the use of a variety of both cell-associated and extracellular virulence factors, some of which are described in the section below.

1.1.5. Virulence factors that cause damage to the host.

The host-pathogen interaction is reducible to two outcomes. That is, those that result in damage to the host causing disease and those that result in no damage to the host and therefore no disease. Thus disease occurs when sufficient damage is sustained to perturb the normal functioning of the host. In this context the word "damage" is then an inclusive term that encompasses damage to cells such as necrosis, apoptosis and malignant transformation and also organ and tissue damage represented by granulomatous inflammation, fibrosis and tumour (Casadevall.A and Pirofski.L 1999).

Damage to the host can be mediated by either the pathogen or the host itself. Often the nature and extent of this damage is modulated by the immune status of the host. Damage in a host that has an insufficient immune response is primarily pathogen mediated where as damage in a host that mounts an abnormally strong immune response is often host-mediated (Casadevall.A and Pirofski.L 1999). However in interactions between pathogens and hosts with a normal immune status, a continuum exists that results in disease only when damage impairs the normal function of the host. Hence disease is one possible outcome of a complex host-pathogen interaction that can arise due to pathogen-mediated damage.

Damage to a host can result partly from the growth of the pathogen that can often produce tissue alterations. However in many cases damage results from the secretion of bacterial exoproducts such as toxins. Most bacterial exotoxins can be classified as either membrane-damaging or those that have an intracellular activity. Membrane-damaging toxins may damage membranes through proteolytic action and in some cases through degradation of components of the lipid bi-layer itself. Other toxins may also be pore-forming or detergent

like causing cell lysis through solubilisation and rupture of the cell membrane (Mims 1997).

Extracellular enzymes called phospholipases are produced by many different pathogenic species. These remove the charged head group from the lipid portion of phospholipids, comprising the eukaryotic cell surface membrane. The charged head group is responsible for the stabilisation of the phospholipid bilayer. Hence the removal of this group results in membrane instability and eventual lysis of the host cell (Salyers 1994).

The most completely studied example of a phospholipase is the α-toxin produced by the anaerobic Gram-positive bacterium *Clostridium perfringens,* the organism most commonly associated with gas gangrene. This has been shown to be a phospholipase C, a metallo-enzyme that cleaves the phosporyl choline head group from phosphatidyl choline and spingomyelin (Mims 1997; Titball 1999).

It is the products of the phospholipid hydrolysis and the production of diacylglycerol or ceramide by the enzyme rather than the overt cell membrane damage, which are responsible for the damage to the host. Diacylglycerol acts as an important secondary messenger within cells through activation of PKC and triggering PKC mediated responses. This includes the activation of platelet-activating factor by endothelial and epithelial cells, which may contribute to local inflammatory responses. PKC also activates eukaryotic membrane-bound phospholipases which hydrolyse membrane bound phospholipids further contributing to cell damage (Titball 1999).

Unlike phospholipases pore-forming toxins insert themselves into the host cell membrane and form channels. The difference in osmotic potential between the extra and intracellular

environment allows the out flux of the cytoplasmic contents of the cell with a concomitant influx of water. Thus the formation of pores causes the cell to swell and eventually lyse due to increased osmotic pressure (Salyers 1994). Pore-forming toxins include the adenylate cyclase hemolysin produced by *B. pertussis* and the staphylococcal δ, α and γ-toxins (Mims 1997).

Many toxins that act intracellularly share a similar mode of action in that they bind to specific receptor on the cell surface membrane are internalised and interact with an intracellular target (Middlebrook and Dorland 1984). Most of these toxins also share common structural features in that they are comprised of two basic subunits. The B subunit mediates binding of the toxin to the cell surface membrane and often facilitates entry of the toxin to the cell cytoplasm. The A subunit is the active component of the toxin, and it is in this that the biological activity resides that confers toxicity (Mims 1997).

Many A-B toxins may be synthesised as a single polypeptide chain. Post-translational modification through proteolysis releases the A portion which can then exert its toxic effect on the cell. Alternatively the toxin may be produced as a more complex form that utilises multiple B subunits which oligomerise on the cell surface membrane and allow entry of the toxin to the cell. It is this B portion of the toxin which binds to specific receptors on the host cell and hence confers the host cell specificity and tissue tropism seen in many toxins (Salyers 1994).

After the binding of the B subunit the toxic A subunit must be internalised to exert toxic activity. This can occur through endocytosis of the bound toxin prior to internalisation of the A portion. Acidification of the endocytic vacuole may further facilitate internalisation by causing structural changes in the A and B portions (Salyers 1994). In other cases

endocytotic processes do not appear to be involved. The A portion in these instances is seen to directly translocate through the host cell's cytoplasmic membrane to its site of action in the cytoplasm.

Once internalised the activity of many bacterial toxins is enzymatic. A common mechanism that these enzymes employ to disrupt cellular pathways is through ADP-ribosylation of endogenous nicotinamide adenosine diphosphoribose (NAD) and attachment of the adenosine diphosphoribose moiety to a cellular substrate such as a host protein (Mims 1997). This in some cases inactivates components of the cellular biosynthetic machinery. It may also cause a marked increase in cellular metabolism which although not deleterious to the cell, has severe over all consequences in animals (Middlebrook and Dorland 1984). A number of bacterial toxins that act through ADP-ribosylation of host proteins have been investigated in detail, some of which are described below.

Corynebacterium diptheriae is the causative agent of the disease diphtheria. The clinical symptoms observed are almost exclusively caused by the production of diphtheria toxin by the organism following colonisation of the epithelial surface of the naso-pharynx (Mims 1997). The toxin is encoded by a β-lysogenic bacteriophage but its expression is controlled by the bacterium in response to iron starvation. Diphtheria toxin is lethal to many animal species and to a wide range of cultured cell-lines. The toxin is disseminated from the site of infection and has deleterious effects on both the heart and nervous system (Middlebrook and Dorland 1984; Mims 1997).

Diphtheria toxin conforms to the A-B paradigm already described with the B-subunit mediating binding to host cells and the A-subunit having an enzymatic activity.

Internalisation of the toxin occurs through receptor mediated endocytosis through clathryn-coated pits. This gives rise to acidified endosomes in which the low pH facilitates escape of the A-subunit into the cytosol. A conformational change of the B-subunit in the acidic conditions allows interaction with the endosomal membrane, which results in the formation of cation channels through which diphtheria toxin A-subunit can escape (Mims 1997). Once in the cytosol diphtheria toxin ADP-ribosylates the cytoplasmic elongation factor EF-2 through binding to a modified histadine residue on the molecule called dipthamide. As EF-2 plays an essential role in protein production the toxin eventually kills host cells through inhibition of protein synthesis (Salyers 1994).

Vibrio cholerae toxin is responsible for the severe diarrhoea seen in patients with clinical cholera. In contrast to diphtheria toxin, the toxin produced consists of five B-subunits and two A-subunits A_1 and A_2. The A_1-subunit posses the enzymatic activity used to ADP-ribosylate the target host protein (Middlebrook and Dorland 1984). Entry of A_1 occurs not through receptor-mediated endocytosis as with diphtheria toxin but is mediated through the five B-subunits and the A_2-subunit after binding to the cell surface gangliside G_{M1} (Mims 1997).

Once internalised the target for cholera toxin is the multi-subunit adenylate cyclase localised on the cytoplasmic side of the plasma membrane, which is responsible for the production of cyclic AMP (cAMP). This is an important second messenger molecule and is responsible for the amplification of cellular responses to external stimuli including hormones. The production of cAMP is controlled by a heterodimeric regulatory complex collectively called G-protein (Mims 1997) ADP-ribosylation of the G-protein by cholera toxin interferes with the proper functioning of the adenylate cyclase causing the over production of cAMP. This causes a number of metabolic defects, among which is the loss

of control of iron flow within the cell. This subsequently results in the loss of water from host tissues, which is seen as the watery diarrhoea characteristic of the disease (Salyers 1994).

Not all bacterial virulence factors cause direct damage to host systems. Some may also cause damage indirectly through the induction of immune responses. This includes the production of inflamatory mediators such as cytokines, thromboxanes and prostaglandins (Salyers 1994). This method of damage is particularly exemplified by the role of lipopolysaccharide (LPS) in Gram-negative bacterial sepsis.

LPS is found predominantly in Gram-negative bacteria and is an integral component of the bacterial outer membrane (Salyers 1994). This molecule is also referred to as endotoxin as the administration of LPS to animal models has a variety of toxic biological effects. Diseases in which endotoxin mediated responses may play an important role include typhoid fever, bubonic plague and brucellosis. It is also considered important in a variety of nosocomial infections caused by Gram-negative opportunistic pathogens particularly after surgery such as *E. coli, Enterobacter* species and *Serratia marcessens* (Mims 1997).

It is the lipid A portion of LPS that is the toxic moiety. The polysaccharide component although not directly toxic is also important for conferring stability on micellar aggregates of LPS. These may form as membrane derived blebs during growth of some species or after cell lysis (Mims 1997; Beveridge 1999). These interact with the host to produce a wide array of biological activities with often very complicated modes of action.

LPS released into the bloodstream is first bound by special plasma proteins called LPS-binding proteins. These LPS-LPS binding protein complexes then interact with CD14

receptors on monocytes, macrophages and with other receptors on endothelial cells. This causes the release of vasoactive substances and activates the alternative complement cascade. It may also activate factor XII that is the first step in the coagulation cascade, which then may result in disseminated intravascular coagulation in the terminal stages of the disease. Many of these reactions are due to the release of cytokines from macrophages and other cells in response to endotoxin. These include IL-1 (IL-1α and IL-1β) IL-6, IL-8, TNF-α and PAF that in turn stimulate the release of protaglandins and leukotriens (Salyers 1994).

Only small quantities of LPS are required to stimulate cytokine release. Production of the endogenous pyrogens IL-1α, IL-1β and TNF-α act on the hypothalamus and give an elevation of body temperature within one hour (Salyers 1994). There is an effect on circulation, leading ultimately to vascular collapse. Different vascular regions are effected depending on species but in man the most noticeable changes are seen to occur in the lungs (Mims 1997). If large amounts of LPS enter the bloodstream vascular pooling may occur along with a drastic fall in blood pressure. Thus as endotoxin enters the blood stream during Gram-negative bacterial sepsis, the vasomotor action of LPS becomes important and the effect is seen systemically as septic shock. This may then subsequently lead to multiple organ failure and death (Mims 1997).

Thus a successful bacterial pathogen has a multitude of capabilities represented by its complement of virulence factors. These are intrinsic to its status as a pathogen and allow virulence. Without the special abilities conferred by these virulence factors a bacterium could not produce disease.

Several brief examples of how some bacterial virulence factors can contribute to disease in the mammalian host have already been given. However the role of these has not been

discussed in relation to particular species that are seen as successful pathogens of humans. To this end, examples of two pathogenic species are given and the interaction of the virulence factors that they use to cause disease are discussed in detail in the following sections.

1.2. *Pseudomonas aeruginosa*: The bacterium and mechanism of virulence

Pseudomonas aeruginosa is a Gram-negative, non-fermentative rod that is ubiquitous in the environment. It is found in soil and wetland habitats as well as associated with plant and animal tissues. Though normally free living, it can cause opportunistic infections particularly in the hospital environment where it has become the pathogen most frequently isolated from individuals who have been hospitalised for more than a week. It is capable of multiplying in eye drops, disinfectants and even in the small pools of water that collect on sinks (Vindenes and Bjerknes 1995). Increasingly *P. aeruginosa* is also becoming an important pathogen in immunocompromised individuals where it can cause a systemic infection, with mortality rate approaching 100%. A common feature of infections involving *P. aeruginosa* is the interruption of the natural barriers that protect from infection, allowing entry of the organism to the body. Unfortunately, *P. aeruginosa* is naturally resistant to many antibiotics often making effective treatment impossible (Prescott, Harley *et al.* 1996).

Cysitic fibrosis (CF) is the most common autosomal recessive trait found in approximately 1in 2000 caucasian individuals. The mutated gene responsible for this disorder has been identified as a transmembrane protein called the CF transmembrane regulator (CFTR). CFTR functions as a chloride ion transporter with mutations giving defects in chloride ion transport. The imbalance of ions leads to the aggregation of very viscous and dehydrated bronchopulmonary and gastrointestinal secretions which block the male sex ducts, the ducts to the pancreas and the airways to the lungs (Nelson, Butler *et al.* 1994). Mucous producing glands continuously secrete, causing them to swell and cysts to form. In the lungs this "sticky" layer impairs the function of the muco-cilliary system, which results in

the inefficient clearance of microorgansims. Also the high sodium concentration in the mucous of CF patients has been found to inhibit the activity of bacteriocidal peptides secreated by respiratory epithelial cells (Saini, Galsworthy *et al.* 1999). The increased susceptibility to bacterial infection often results in a persistent colonisation, which in turn initiates chronic inflammatory responses. This chronic microbial colonisation of the airway by a number of different bacterial species and in particular *P. aeruginosa*, is the major cause of mortality in CF patients, accounting for up to 90% of deaths.

The genome of *P. aeruginosa* has been sequenced and found to be larger than most of the other bacterial genomes so far sequenced. At 6.3 Mb and 5,570 open reading frames (ORFs) the genetic complexity of this opportunistic pathogen approaches that of a simple eukaryote (Hancock 1998; Stover, Pham *et al.* 2000). Of all the sequenced bacterial genomes to date *P. aeruginosa* has the highest proportion of regulatory genes. This includes a large number of genes in the LasR, AraC, and ECF-σ groups and two-component regulator families (Hancock 1998). These systems permit organisms to respond to environmental changes rapidly and are often associated with the regulation of virulence. Hence the increased environmental versatility and pathogenic potential observed in *P. aeruginosa* is the product of a high degree of genetic complexity.

1.2.1. Virulence factors produced by *P. aeruginosa* and their role in disease.

P. aeruginosa produces a range of cell-associated and extracellular products, some of which have been identified as key virulence determinants. These include pili, exoenzymes, siderphores, *Pseudomonas* exotoxin A, TTS systems and EPS (Vindenes and Bjerknes

1995). These may work synergistically or along with the hosts own immune response to cause damage to infected tissues.

Adherence of *P. aeruginosa* to cell surfaces is mediated through the use of a class of protein adhesins called type IV pili. These are also involved in twitching motility and have been implicated in a form of social movement called swarming. Type IV pili have also been shown to be essential in the formation of biofilms on both biotic and abiotic surfaces (Bellido, Martin *et al.* 1992) and so may mediate initial colonisation of the lung, as biofilm formation is essential for persistent colonisation of respiratory tissues. Additionally a decrease in virulence is observed when the genes involved in pili bisynthesis are inactivated through mutation, indicating their importance in the virulence of this organism.

In its late logarithmic to stationary phase *P. aeruginosa* is capable of secreting high levels of the degradative enzyme elastase. This elastolytic activity of *P. aeruginosa* has been shown to be important in disease, particularly in lung infections, as up to 30% of the total protein in the lung is comprised of elastin that allows the lung to expand. The elastolytic properties stem from the co-ordinated activity of two enzymes, LasA and LasB. LasA, a serine protease, nicks the protein probably causing it to denature, allowing LasB, a zinc metalloprotease to degrade it. The elastolytic activity is likely to be the major cause of the direct damage and the haemorrhaging observed in *P. aeruginosa* infections of the lung. The proteins may also aid in the establishment of infection and aid in the avoidance of non-specific immune responses due to their ability to degrade components of the complement cascade (Prescott, Harley *et al.* 1996).

The acquisition of iron by *P. aeruginosa* involves the secretion of two chemically unrelated siderophore molecules, pyoverdin and pyochelin. The receptors for ferri-

pyoverdin and ferri-pyochelin have also been well studied. In addition to the characteristic negative regulation by iron concentration mediated indirectly through the Fur repressor, expression of the receptors is also positively regulated by the production of corresponding siderophore (Meyer, Neely *et al.* 1996; Poole 1996).

Pyoverdins (also called pseudoactins) have been shown to be the most important siderophore produced by *P. aeruginosa* (Meyer, Neely *et al.* 1996). They are characterised by a conserved hydroxyquinolone moiety called the chromophore and an amino acid tail of variable length. The differences in this side chain are used to differentiate the various pyoverdins produced by the pseudomonads and are likely to be responsible for the observed specificity of pyoverdin utilisation by individual strains of *P. aeruginosa* (Poole 1996). The siderophore is capable of removing transferrin-bound iron in vitro which may contribute to the organism's ability to grow in human serum (Meyer, Neely *et al.* 1996). Pyoverdin as also been shown to be required for the colonisation of the lung in a rat model of infection (Choi 2002). Pyoverdin is then important for iron acquisition and growth in the lung environment and thus likely contributes to the chronic pulmonary *P. aeruginosa* infections seen in those patients with CF (Poole 1996).

Unlike pyoverdin, pyochelin only exhibits low affinity for iron *in vitro*. However the siderphore has been shown to promote bacterial growth and lethality when injected into the peritoneal cavity of mice along with avirulent mutant strains of *P. aeruginosa* (Choi 2002). It may also contribute to virulence through the production of free radicals and damage of endothelial cells. Thus its role in the virulence may not be related to the acquisition of iron *in vivo*.

Pseudomonas exotoxin A has the same cellular activity as diphtheria toxin in that it inhibits protein synthesis by cleavage of NAD+ and ADP- ribosylation of elongation factor (EF) 2 (Mims, Dimmock *et al.* 1997). However the two proteins differ antigenically and cross reactivity of neutralising antibodies has not been reported. Different receptors on the cell surface are also used by the toxin subunits to facilitate entry to the cell through receptor mediated endocytosis. Originally LPS was thought to be the most toxic component associated with *P. aeruginosa* but *Pseudomonas* exotoxin A has since been reported to be 20,000 times more toxic than LPS in animal studies. Mutants deficient in *Pseudomonas* exotoxin A production show reduced pathogenicity, indicating a direct role in virulence (Bodey, Bolivar *et al.* 1983; Whitt and Salyers 1994). Furthermore the toxin has also been shown to contribute to disease process through tissue damage and reduction of the bactericidal activity of macrophages and other phagocytic cells (Whitt and Salyers 1994).

An important virulence determinant of *P. aeruginosa* is the TTS mechanism. This has been shown to be present in almost all clinical and environmental isolates of *P. aeruginosa* (Feltman, Schulert *et al.* 2001). Currently four effector proteins, secreted through the *P. aeruginosa* TTS system have been identified. These include ExoS, ExoT, ExoU and ExoY (Fink-Barbancon 2001). ExoS belongs to the family of ADP-ribosylating toxins that include cholera toxin and pertussis toxin, which are capable of ADP-ribosylation of a number of host proteins such as Ras and Rab. This effects vesicular traffic within the cell resulting in eventual cell death. For full enzymatic activity ExoS must first be activated by a eukaryotic 14-3-3 protein (Barbieri 2001). After activation, the enzyme is cytotoxic to eukaryotic cells and has been reported to play a role in disease caused by this organism (Barbieri 2001).

The amino-terminus of ExoS contains a GTPase-activating domain for Rho GTPases. This induces cell rounding through actin rearrangements within target cells (Feltman, Schulert *et al.* 2001). Thus ExoS may also play a role in disease through the modulation of bacterial internalisation into cells (Feltman, Schulert *et al.* 2001). Studies suggest that it may disrupt epithelial cell signal transduction pathways, resulting in breakdown of the epithelial barrier, hastening the onset of disease (Goranson, Hovey *et al.* 1997). It can also impair phagocytosis by affecting both the cytoskeleton-engulfment process and phagosome maturation (Barbieri 2001).

The other effector molecules of the *P. aeruginosa* TTS system have so far been poorly characterised. ExoT also like ExoS has an ADP-ribosyltranferase activity. It is also a GTPase activating protein that is capable of inhibiting bacterial internalisation by eukaryotic cells (Feltman, Schulert *et al.* 2001). The role of ExoY in the pathogenesis of *P. aeruginosa* has not yet been fully determined but it has been shown to be an adenylate cyclase that is capable of elevating intracellular cAMP levels. Likewise the mechanism of action of ExoU has not been defined. The enzyme mediates the killing of a wide range of mammalian cell types in as little as 3-4 hours of the initial infection. These include macrophages, epithelial cells and fibroblasts. In animal models of lung infection the delivery of ExoU by *P. aeruginosa* causes acute cytotoxicity and appears to play a major role in the septic shock caused by the organism. It may also aid in evasion of the host responses by decreasing macrophage viability and phagocytic function (Fink-Barbancon 2001).

An interesting and extensively studied virulence factor of *P. aeruginosa* is the production of an exopolymer comprised of mannuronic and guluronic acid called alginate. Conversion to the alginate producing phenotype is often associated with a chronic persistent

colonisation of the lung in CF and signifies a poor prognosis for the patient, as infection is seldom eradicated even with aggressive antibiotic therapy (Govan and Deretic 1996).

Alginate surrounding the bacterium or as a free substance may contribute to the pathogenesis of the organism in several ways. Alginate surrounding the cell may serve as a direct barrier against phagocytic cells and effective opsonisation by antibodies. It is also capable of quenching reactive oxygen species and scavenging the hypochlorite molecules, important in the bactericidal ability of macrophages (Govan and Deretic 1996).

Alginate also plays a role in the adhesion of *P. aeruginosa* to biotic surfaces such as the respiratory epithelia. Alginate may act as a non-pilus adhesin aiding in the formation of the characteristic microcolony containing biofilm found in the lungs of CF patients. Furthermore, in these and other affected individuals alginate may have imunomodulatory effects through suppression of lymphocyte function and stimulation of the production of the proinflamatory cytokines IL-1 and TNF-α. Alginate also stimulates the release of IL-8 from epithelial cells, which possibly contributes to the recruitment of neutrophils, which dominate the inflammation of the airways in CF (Govan and Deretic 1996).

Although alginate only plays a limited role in the primary adhesion of the organism it does directly contribute to the persistence of the organism in the lung. It is likely that alginate interferes with the action of antibiotics through mechanical restriction of antibiotic penetration to the cell. Thus contributing to the inability of mucoid *P. aeruginosa* strains to be eradicated from the lung even with very aggressive antibiotic therapy (Govan and Deretic 1996).

P. aeruginosa is an important pathogen that is responsible for a variety of life-threatening conditions largely in immunocompromised populations. However other related genera also

have the capacity to cause disease in humans. This is particularly exemplified by some members of the *Burkholderia* genus. These are capable of causing severe disease in the immunocompromised but importantly also in apparently healthy individuals.

1.3. *Burkholderia pseudomallei*: The bacterium and mechanisms of virulence.

Burkholderia pseudomallei, formerly *Pseudomonas pseudomallei* is an environmental saprophyte that is capable of causing a wide spectrum of disease in a variety of hosts including humans, dolphins, sheep and pigs which is collectively known as melioidosis (Ellis 1999). Although very similar to *Burkholderia cepacia* in both biochemical and chemical characteristics (Kanai 1994) *B. pseudomallei* is considered the more dangerous pathogen as it causes a more severe disease in humans. It has also recently been described as an emerging pathogen that could represent a potential global problem as its prevalence increases (Dance 2000).

Melioidosis was first described by Whitmore in 1913 after observation of a previously undescribed disease affecting the population of the Indian city of Rangoon (Whitmore 1912). It was not until the late 1950's when investigators began to understand that both man and animals were likely to be exposed to the organism through contaminated soil and water (Dance 2000). Later, attention was focused on the organism as a consequence of the military operation of American soldiers in the Vietnam War. A disproportionate number of helicopter crewmen were reported to develop pulmonary melioidosis. This was thought to be caused by inhalation of contaminated dusts raised by helicopter rotor blades. This prompted further research into the ecology and distribution of *B. pseudomallei* and the prevalence of melioidosis in endemic areas (Kanai 1994).

In the environment *B. pseudomallei* is a free-living soil saprophyte, which is found in tropical areas from 20 °N to 20°S of the equator. It is isolated most frequently in China (Yang 2000), Africa, the Indian subcontinent, Iran, sub-Saharan Africa, Northern

Australia, Papua New Guinea (Currie, Fisher *et al.* 2000) and Central and South America (Dance 2000). These consist of two main serotypes classified on the basis of thermostable and thermolabile antigens. Serotype 1 possesses both types of antigen and is found predominately in Asia. However serotype 2 has only themostable antigens and is found in Australia and Africa (Yang 2000). The increase in world travel to areas of endemicy has also increased the isolation rate of *B. pseudomallei* and cases of melioidosis have now been recorded in Europe (Kanai 1994).

Sites most likely to contain *B. pseudomallei* are cleared cultivated or irrigated agricultural sites such as sports fields, animal paddocks and rice paddies. In endemic areas such as Northeast Thailand where melioidosis is common, the number of *B. pseudomallei* per gram of soil is up to 20 fold higher than in other non-endemic areas (Dance 2000). In these areas, those in contact with rice paddies may be at particular risk of *B. pseudomallei* infection as many of the conditions found in wet rice paddies favour the growth of the organism. The optimum temperature for growth of *B pseudomallei* is 37- 42 °C and at a pH between 5.0 and 8.0. These conditions are found in rice fields of Northeastern Thailand which is covered in mostly acidic soils (Kanai 1994). Within the rice paddy the action of nitrifying and denitrifying bacteria allows anaerobic conditions to form. As *B. pseudomallei* is a facultative anaerobic organism which can obtain energy through nitrate reduction in anaerobic environments the conditions encountered seem ideal for the pathogen's growth (Kanai 1994). The application of nitrate fertilisers to rice fields may also contribute to the organisms proliferation in agricultural land as the addition of supplementary nutrients to the environment may promote its growth (Dance 2000).

There is a strong correlation between rainfall and the incidence of *B. pseudomallei* infection, as 75% of cases are reported during the rainy season (Dance 2000). The isolation

rate of *B. pseudomallei* is highest from clay soils. Bacteria and water are thought to be attracted to clay particles through electrostatic interactions. This retains moisture that would normally be lost in the dry season, possibly contributing to the organism's persistence in endemic areas. The rise in the water table in the rainy season would then carry bacteria to surface waters increasing the chance of exposure (Ellis 1999). However this seasonality of infection may also reflect the increased exposure to the organism during rice harvesting and planting which occur at the wettest times of the year.

It has been reported that approximately 80% of the population in Northeastern Thailand are sero-positive for antibodies to *B. pseudomallei* by the age of 4 years old (Ellis 1999). Also in these areas *B. pseudomallei* is responsible for 20% of the community-acquired septicaemia observed in the indigenous population (Chaowagul, Suputtamongkol *et al.* 1993; Brett and Woods 2000). Thus *B. pseudomallei* infection represents a major health concern to both rural and urban communities in endemic areas.

Infection is thought to occur through one of several possible routes. These include the inoculation of organisms through penetrating wounds or through existing skin lesions (Brett and Woods 2000). The aspiration of contaminated water during near drowning episodes, iatrogenic inoculation and laboratory acquired infection which result in melioidosis have also been shown to occur. There are no reports of animal-to-human spread but person-to-person spread of *B. pseudomallei* has been documented twice (Dance 2000). Arthropod-borne infection has also been reported to occur although this has only been demonstrated under laboratory conditions (Ellis 1999). There are no well documented cases of infection through ingestion although experimental animals can be infected through this route (Dance 2000).

Disease, after a usual incubation period of 2-3 days, may lead to one of four of different clinical outcomes which include acute fulminant septicaemia, sub-acute illness, chronic infection and sub-clinical disease (Ellis 1999). The disease may be disseminated throughout the body or localised to a particular organ. However pathology most commonly occurs in the liver, spleen and lung (Brown, Boddy *et al.* 2002).

The acute septicaemic form of melioidosis is characterised by meningitis with cutaneous and sub-cutaneous lesions and may be rapidly fatal. This form of the melioidosis is usually associated with an additional underlying disease or condition (Haase, Janzen *et al.* 1997). The most common predisposing factors are diabetes mellitus, chronic renal disease, alcoholism, malignancy, connective tissue diseases, HIV and steroid therapy (Dance 2000) (Ellis 1999; Brett and Woods 2000; Ulett, Currie *et al.* 2001). These conditions lead to immune deficits including phagocytic defects, diminished humoral responses and diminished cytokine production which possibly contribute to the increase in susceptibility observed (Dance 2000).

A second form of the acute disease is pulmonary melioidosis. This has been reported to be the second most frequent form of disease presentation and is often mistaken for tuberculosis (Kanai 1994). The disease is characterised by a high fever, respiratory distress and the appearance of visceral abscesses. As with the septicaemic form, if left untreated death often occurs within days and in severe cases in as few as 48 hours (Haase, Janzen *et al.* 1997). Both forms of acute disease have a very high mortality rate of approximately 50-75% (Ellis 1999) and death may occur even if prompt and aggressive antimicrobial chemotherapy is carried out.

The sub-acute form of the disease is characterised by a prolonged fever with multiple abscesses on the viscera. In the latter stages of the disease bacteria can be isolated from blood, pus, urine and other bodily secretions. This may then represent a possible mechanism for environmental dissemination of the organisms to non-endemic areas. Death from the sub-acute disease may occur in a few days or after months if the condition is left untreated (Leelarasamee and Bovornkitti 1989). This is unlike the sub-clinical form where death only occurs on activation of the disease by a traumatic event. It may be even left undiagnosed only to be detected upon post-mortem examination of tissues (Brett and Woods 2000).

Melioidosis with neurological involvement without meningitis only occurs in a small percentage of cases. The disease affects the brainstem and spinal cord and can cause facial palsy and motor weakness similar to that seen in Guillian-Barrė syndrome, a progressive demylination of the motor neurones (Currie, Fisher *et al.* 2000). A number of patients with neurological melioidosis have been reported to also have perforated eardrums. Thus entry through the damaged timpanic membrane in these individuals may be a further source of inoculation, through exposure to contaminated water (Currie, Fisher *et al.* 2000).

Although *B. pseudomallei* can cause severe and often rapidly fatal illness it may also form latent infections in infected individuals. These can then recrudesce to give disease anything up to 26 years following the initial exposure to the organism (Mays and Ricketts 1975; Brett and Woods 2000). The relapse of latent infections has previously been associated with recovery from acute *B. pseudomallei* infection. Analysis of 16s ribosomal DNA (ribotyping) of organisms isolated from patients with reoccurring disease has shown that almost all cases of reinfection are actually relapses caused by the original infecting strain (Chaowagul, Suputtamongkol *et al.* 1993; Ellis 1999; Jenney 2001).

In one study the reported median time to relapse was approximately 21 weeks with the chance of relapse falling with a longer disease free interval (Chaowagul, Suputtamongkol *et al.* 1993). It has also been shown that a number of factors may influence the likelihood of relapse. These include the severity of the original disease presentation and the level of dissemination of the organism throughout the patient. Other factors thought to contribute to the relapse of latent infections include the invasion and survival of *B. pseudomallei* within privileged sites such as phagocytic cells and the persistence of the organism within non-phagocytic cells (Brown, Boddy *et al.* 2002). Large numbers of organisms are also found in the sealed off abscesses that are characteristic in the pathology of melioidosis. This may represent another potential source of reinfection and relapse of disease in previously infected individuals (Chaowagul, Suputtamongkol *et al.* 1993). Relapse is reported to be much more likely in those who have had systemic disease. The choice of initial antimicrobial treatment may also be an additional risk factor, as appropriate treatment can reduce the risk of relapse by half (Chaowagul, Suputtamongkol *et al.* 1993). Additionally, although as already described acute melioidosis is more prevalent in those with pre-existing medical conditions such as diabetes, no such link with underlying disease and the chance of relapse has been described.

1.3.1. Virulence factors produced by *B. pseudomallei* and their role in disease.

B. pseudomallei produces a wide variety of extracellular and cell-associated virulence factors. These include proteases, lecthinases, lipases, siderophores and putative toxins that have a cytolethal or cell elongating activity. Suggested cell associated virulence factors include pili, LPS, flagella and an EPS capsule (Ellis 1999; Corbett 2003). These presumably allow the organism to colonise a potential host and persist intracellularly within the body while also allowing avoidance of the host immune response as in other pathogenic species. The precise role of these virulence determinants in the pathogenesis of melioidosis is not fully understood and many remain poorly characterised (Ellis 1999). However the release of the completed *B. pseudomallei* K96243 genome from the Welcome Trust's Sanger Center (http://www.sanger.ac.uk/Projects/B_pseudomallei/) will underpin new studies concerning the pathogenesis of *B. pseudomalle*i and provide valuable missing information regarding the molecular basis of the pathogenicity of this organism in the mammalian host.

A number of biochemical, immunological and molecular approaches might be taken for the identification of *B. pseudomallei* virulence factors that are important in disease. A selection of these techniques are described in the following sections.

1.4. Approaches to the identification of bacterial virulence factors.

Before the advent of accepted scientific methodology and modern techniques the investigation of bacterial virulence was limited to the determination of an organism's ability to cause disease. This was achieved through the use of a set of postulates formulated by Koch in the 1800s. These stated that a causal link could be drawn between a particular organism and a disease if; (1) the bacterium was consistently found in those with the disease or its products found in parts of the body affected by the disease. (2) The bacterium could be isolated from lesions and maintained in culture. (3) Re-administration of the organism to a susceptible host should produce symptoms of disease. (4) Finally the same bacterium should be re-isolated from the infected host. However due to the inability to culture some pathogenic organisms and subsequent advances in technology coupled with a deeper understanding of bacterial pathogenicity Koch's postulates became too restrictive (Salyers 1994). Thus a new view of the investigation of bacterial pathogenesis was taken based on investigation of host-pathogen interactions using more powerful techniques.

1.2.1. Classical methodologies.

Before the advent of molecular biological methods and genome sequencing, technologies for the identification of bacterial virulence factors were limited to biochemical and immunological methods. Biochemical methods relied principally on the purification of a molecule suspected of mediating a particular host-pathogen interaction and studying its action in both *in vitro* and *in vivo* systems (Salyers 1994). This type of approach allows

detailed information to be obtained about the physical properties of a molecule, which would not be available any other way. However, this method has two important limitations. Firstly, the molecule being studied must have an measurable activity. The biochemical approach is then most useful when the identity of a bacterial virulence factor is known or suspected. A second limitation of the biochemical approach is that measurements on a purified molecule *in vitro* may not reflect its activity *in vivo*. To prove function *in vivo,* an immunological approach must be taken (Salyers 1994).

The immunological approach to the identification of a bacterial virulence factor was to determine whether antibodies to a bacterial product were protective in animals infected with the bacterium from which the product originated. If protection was seen, the bacterial product can be assumed to contribute to virulence. However antibodies to bacterial surface components may allow protection from infection through opsinisation of bacterial cells or through the enhancement of complement mediated killing. Thus a link between the production of antibodies to a particular virulence factor and the pathogenicity of a given organism cannot be made, without information given through the use of the biochemical techniques already described.

The use of specific antibodies to bacterial virulence factors has also allowed the development of other technologies that have been useful in the isolation and confirmation of suspected virulence factors. This includes immunoelectron microscopy. This method allows the direct observation and localisation of bacterial products in infected host tissues, through binding of specific antibodies carrying a marker detectable through the use of electron microscopy. Also techniques such as enzyme-linked immunosorbent assay (ELISA) have been developed. This method relies on specific antibodies to a known or

suspected virulence factor and can be used to monitor levels of particular bacterial products through out the course of an infection (Quinn 1997).

The use of these classical techniques has provided a wealth of information about a variety of bacterial virulence factors and their role in disease. However the process of identification was slow, and often limited to products that had an obvious role in disease such as toxins or specific adhesins. This limitation was addressed through the advent of modern genetic methods. These have allowed the development of new approaches to the identification of bacterial virulence factors. This has lead to a re-definition of Koch's postulates of disease. This new set of criteria is called the molecular Koch's postulates and gives three conditions that must ideally be met to allow identification of genes involved in virulence. These are: (1) specific mutants deficient in the phenotype of interest must be isolated, (2) the wildtype virulence gene must be cloned and (3) the wildtype phenotype must be restored through re-introduction of the wildtype gene into the mutant (Quinn 1997; McDaniel 2000).

The wide availability of molecular biological methods and the relative ease with which these can be used to address the molecular postulates of disease has lead to the development of newer technologies that rely on three basic approaches. This includes mutagenesis, complementation analysis in avirulent strains and analysis of differential expression of genes in response to the host environment. However, although a wide variety of molecular biological methods now exist based on these themes, to acquire accurate information regarding the virulence mechanisms used by human pathogens through these methods, a model system utilising animals or parts of animals inevitably must be used (Quinn 1997).

1.4.2. Animal models of bacterial infection.

Due to ethical constraints, humans can rarely be used as a model system to study bacterial pathogenesis. Therefore in order to identify virulence determinants experimental models that mimic the specific environment of the natural host are required (Quinn 1997). A variety of model systems have gained acceptance as appropriate for the study of human disease. Although not an exhaustive this list includes non-human primates, guinea pigs, mice and genetic variant mice. It also includes other less commonly used animal species such as hamsters, rabbits, dogs and the nine-banded armadillo.

The mouse model has become the most commonly used small animal model for microbiological research (Houpikian 2002). A wide variety of different wildtype mouse strains have been created through selective breeding of desirable characteristics with subsequent inbreeding to maintain the phenotype in the population. Large numbers of spontaneous and radiation induced mutations are also available. This allows for differential responses to particular pathogens to be induced and has given insights into many disease processes. Isogenic knock-out strains of mouse are also available. In these mice, defined mutations in targeted genes of the germ line give rise to selectable phenotypes (Rossant 1998). These may resemble inherited genetic disorders such as CF (Davidson, Dorin *et al.* 1995) and may also give rise to other phenotypes useful in the investigation of bacterial pathogenicity. This includes the lack of an immune system or a down regulation of a part of the immune system, such as the lack of a particular cytokine or cell-surface receptor (Kuhn 1991).

Non-human model systems may have considerable disadvantages with regard to the logistical burdens experienced during animal husbandry. Also, safety concerns when working with animals infected with dangerous pathogens such as *B. pseudomallei* must be addressed. However, without the use of these animal model systems many modern techniques for the identification of bacterial virulence factors described in the following sections would not be possible.

1.4.3. Mutagenesis.

One of the earliest and most widely used techniques for the investigation of bacterial virulence involved the creation of mutants in the pathogen of interest. In the past this has been achieved through the use of changes in culture conditions or DNA damaging agents such as chemicals and irradiation (Quinn 1997; McDaniel 2000). When used in conjunction with enrichment strategies (McDaniel 2000) for particular mutations this approach has proved particularly powerful. However the limiting factor in this approach is that the gene in which the mutation occurs cannot be easily identified. This limitation was addressed with the discovery of transposons, which have revolutionised the investigation of bacterial pathogenicity.

Transposons were identified some 60 years ago and are defined as mobile genetic elements that can move within the bacterial genome and integrate randomly (Salyers 1994; Lehoux 2001). Transposons commonly carry a selectable marker such as resistance to a particular antibiotic. This makes the selection of mutants simple. The position of the transposon can be determined and this facilitates cloning of the genes of interest (Salyers 1994). Thus

transposon mutagenesis has become one of the most commonly used and inexpensive tools for the molecular-genetic study of bacterial virulence.

Although very effective with considerable advantages over the other methods described, transposon mutagenesis has disadvantages. Potentially, libraries of thousands of mutants are created each with a transposon insertion in a single gene. To identify mutations that affect virulence, each mutant must be screened in a suitable host. However to screen the entire genome of a pathogen a prohibitively large number of animals would be required. This problem was first addressed by Hensel *et al* (Hensel, Shea *et al.* 1995) who described a modification of transposon mutagenesis method called signature-tagged-mutagenesis (STM). This allows multiple transposon mutants to be screened in the same animal simultaneously for their inability to survive *in vivo*. This is achieved through the tagging of each transposon with an 80 base pair (bp) tag, which has a variable central portion and constant flanking regions. The unique tag then allows the detection of a given mutant through DNA-DNA hybridisation (Fuller, Kennedy *et al.* 2000). Briefly a pool of mutants is used to infect an animal. At an appropriate time post infection the bacteria are recovered. The tags in the recovered pool are then amplified using the polymerase chain reaction (PCR) and used to probe an array consisting of all the tags present in the innoculum. Mutants displaying an attenuated phenotype are seen as those that are absent from the output pool as shown by a lack of binding on the array (Foulongne, Bourge *et al.* 2000). Although originally developed for the study of *Salmonella enterica* serovar Typhimurium pathogenesis, STM has also subsequently been used to identify virulence factors in other bacterial species including *V. cholerae* (Chiang and Mekalanos 1998), *Brucella suis* (Foulongne, Bourge *et al.* 2000), *Yersinia enterocolitica* (Darwin and Miller 1999), *B. pseudomallei* (Atkins 2002), *P. aeruginosa* and *Mycobacterium tuberculosis* (Fuller, Martin *et al.* 2000).

1.4.4. Complementation analysis.

Mutation of bacterial pathogens and screening the resulting mutant library for mutations in virulence related genes, represents only one approach for the identification of bacterial virulence factors. Another method for identifying virulence determinants is through complementation analysis.

Complementation analysis requires the identification of congenic pairs of virulent and avirulent strains and tools that allow transfer of genes from the virulent strain to its avirulent counterpart. Putative virulence genes can be isolated from virulent strains and transferred to the avirulent strain. If transfer confers virulence, a role in pathogenicity can be shown for the selected gene (Quinn 1997). However this approach requires that the cloned gene has a known or suspected function. Furthermore standard cloning procedures using plasmid vectors are limited in that they isolate only small portions of the bacterial genome less than 30 kb (Salyers 1994). Therefore, this method is most successful if only single or a few linked genes are sufficient to induce a virulent phenotype.

This limitation is addressed through the use of different cloning vectors such as cosmids. A cosmid vector is formed from components of plasmids and bacteriophage λ. In addition to the antibiotic selection markers and origin of replication (*ori*) commonly used in plasmid vectors, cosmids also contain one or more cohesive-end sites (*cos*) (Ehrich 1987). This allows recognition of the vector and its insert by the packaging system of the λ bacteriophage. Using this method relatively large segments of DNA, up to 42 kb can be packaged into phage heads. These can then be used to transfer cosmids, through transfection , into a suitably competent *E. coli* host (Collins and Hohn 1978).

By repeating this process with fragments of DNA from a pathogen, ordered libraries representing the genome of the organism, housed in thousands of individual cosmid clones, can be created. These clones can then be propagated and used for investigation of the function of the genes contained with in the cosmid insert. If the inserted sequence confers an increase in virulence to the otherwise non-pathogenic host, the genes contained within the cosmid insert may then be considered as potential virulence factors.

This approach has proven valuable for the investigation of virulence in a variety of pathogens including *Salmonella enteritidis* (Park 1997), *B. pseudomallei, Burkholderia mallei* (Abaev 1997), *V. cholerae* (Connell 1995) and *P. aeruginosa* (Franklin 1993). It continues to be an important adjunct to other commonly used techniques, allowing an insight into the genetic basis of virulence.

1.4.5. Differential expression analysis.

The methods discussed so far have focussed on the search for virulence associated genes through an approach involving the creation of mutations or the expression of cloned genes in heterologus host species. These methods although very powerful are not specific for genes only expressed *in vivo*. Other methods have been subsequently developed that allow the enrichment of genes specifically active during infection.

Bacteria generally express genes when this benefits survival. Accordingly the expression of a subset of genes *in vivo* is necessary for a pathogen to colonise, survive, replicate and cause disease (McDaniel 2000). This principle has been exploited in several strategies for the identification of candidate virulence genes, based on their preferential expression

within animals or under laboratory conditions that mimic the environment within the host. Some of these methods entail the isolation of mRNA, others involve the identification of promoters with host-specific activity. Once mRNAs or promoters are identified, the gene itself can then be identified by using a short segment of the corresponding amino acid or nucleotide sequence and DNA sequencing. This can be compared to genomic DNA sequence databases or used as a probe to clone the gene from an unsequenced genome, facilitating investigation of the genes of interest (McDaniel 2000).

An example of a technique that utilises host-mediated selection of genes expressed during infection is *in vivo* expression technology (IVET). IVET was first described for the identification of virulence-related genes in the Gram-negative organism *S. enterica* serovar Typhimurium (Lowe 1998). In the IVET system, a promoter fusion library is constructed in which cloned promoters direct the expression of a gene that complements a defect in an auxotrophic mutant of the host strain, along with a selectable marker. When introduced into a suitable model, only promotors which are active *in vivo* are identified (Quinn 1997). Bacteria which also show complementation *in vitro* are discarded. Using this method several *S. enterica* serovar Typhimurium *in vivo* induced (*ivi*) genes have been isolated that are expressed exclusively during murine infection (Quinn 1997).

Although now adapted for use in many other Gram-negative and Gram-positive organisms, including *Yersinia* and staphylococcal species, many of the genes that have so far been isolated using IVET have been so called "house-keeping" genes that are involved in general biosynthetic processes or transcriptional regulation (Valdivia 1997). However these genes may also be of interest as potential virulence factors active during the disease process. Hence IVET is considered to be an important strategy for the identification of

bacterial virulence determinants and continues to be applied to the investigation of a variety of pathogenic species.

Other methods of differential expression analysis have also been developed. Differential fluorescence induction (DFI) is a semi-automated method that allows the isolation of conditionally active promoters through fusion to the green fluorescent protein (GFP) (Valdivia 1997). GFP originally isolated from the jellyfish *Aequorea victoria,* when excited with blue light fluoresces green. This signal can then be detected by a fluorescence activated cell sorter (FACS). Thus promoters corresponding to many potential virulence related genes, can be rapidly isolated. When compared to IVET, DFI has yielded a high proportion of virulence genes relative to housekeeping genes (McDaniel 2000).

The majority of genome wide studies of *in vivo* gene expression has used techniques such as IVET and DFI (Mangan 2002). Although these procedures have lead to the identification of putative virulence factors, they are time consuming, technically challenging and difficult to apply to many genetically intractable organisms. Moreover they only partially probe the genome of a given pathogen for virulence related genes. However, new technologies have emerged to monitor bacterial gene expression at a whole genome level during host-pathogen interactions. One such technology is microarray analysis (Mangan 2002).

Using microarrays it is possible to identify genes that are preferentially expressed under the environments of interest or at particular time points throughout the infection process (McDaniel 2000). Genes that are up regulated under each given condition can then be assumed to be important for survival or persistence of the organism (Moxon 2002).

Much of the interest in bacterial whole genome microarrays lies in the identification of gene expression profiles associated with a particular environmental condition or stage of infection. In this way a correlation between expression and function may be derived for many of the genes of bacterial genomes with no known function, some of which may be involved in virulence (Mangan 2002). The potential of microarray technology is great and it will continue to provide information concerning the interplay of genes needed by pathogenic organisms to cause disease. However, the other more traditional techniques described here, will still be required to elucidate the contribution of individual genes to pathogenesis and disease.

The techniques described in this section merely identify candidate virulence genes. These cannot be considered as true virulence related genes until they have met further criteria to satisfy the requirements of the molecular Koch's postulates. In many cases this can only be achieved through the use of a suitable model system such as animals.

1.5. Alternative animal models of bacterial infection.

Animal models utilising higher order organisms remain the gold standard for the *in vivo* investigation of bacterial pathogenicity. However, in today's socio-political climate the use of animals has become undesirable and the minimisation or elimination of the use of animals in research is actively encouraged (Smith and Oyston 2002). Thus, the scientific community has adopted the ideals of the three R's approach to animal experimentation first proposed by Russel and Birch more than 40 years ago (Rowan 1980). The reduction, replacement and refinement of existing animal models has driven the development of alternative technologies for the substitution of animals in science.

An alternative model of infection is defined as one that does not use higher order mammalian species such as mice, rabbits or non-human primates. Some alternative models use cell culture techniques. This allows experimental investigations of tissue tropism, colonisation and tissue destruction, particularly when relating to human pathogens which are often not possible to perform in whole animal models. Thus, when identifying bacterial genes required to establish human disease, *in vitro* model systems containing human cells may be more appropriate (Quinn 1997). However, cell culture systems generally utilise monolayers of cells attached to an artificial substrate and so have significant limitations in the study of bacterial pathogenesis. The most obvious of these being that they are artificial systems and do not represent the complex interactions that occurs in a whole animal.

This limitation is addressed through the use of alternative animal models of bacterial infection. These may use insect, vertebrate or non-vertebrate hosts in place of mammals. This partly ameliorates the various ethical and legislatory problems involved with the use

of experimental animals. It also allows large assays to be carried out and the host-pathogen interactions occurring at each stage of the infection can be studied at the molecular level. This can be achieved through the creation of mutants in host and pathogen, which are both generally amenable to genetic analysis. All this can be done without consideration for the numbers of individual animals being used and at a fraction of the cost of that of classical animal models. Importantly some of those virulence genes found to be important in the pathogenesis towards alternative models of infection have also been found to be essential in disease of classical mammalian models, thus showing their value for the investigation of bacterial pathogenicity (Tan 2002).

1.5.1. Fish

Currently the only alternative vertebrate host that has been used for the investigation of bacterial pathogenicity is the Zebra fish, *Danio rerio*. Most alternative model organisms currently in use lack defence systems important in mammalian host-pathogen interactions including leukocytes, innate cellular immunity and adaptive immune responses. In contrast the zebra fish has a well-developed immune system that is similar to that of mammals. This includes the ability to produce immunoglobulin, antigen processing cells, T cells and B cells as well as the possession of leukocytes capable of producing reactive nitrogen and oxygen species (Neely 2002).

Due to the availability of standard genetic techniques for manipulation of zebra fish genes, its small size and rapid generation time the zebra fish has become an important model for the investigation of vertebrate development. Ongoing genomic sequencing has also identified numerous orthologues of human genes. This suggests that the zebra fish can be

used in the functional analysis of these genes as models of human disease (Neely 2002). Although originally considered as a model of vertebrate development the zebra fish has now been used as an alternative model for the study of strains of streptococci pathogenic to both fish and humans.

Streptococcus iniae is a pathogen of both fish and mammals, originally identified from subcutaneous abscesses on Amazon fresh water dolphins (Neely 2002). In fish the most common presentation of *S. iniae* infection is meningoencephalitis. Systemic invasive infection is also common which resembles some streptococcal infections of humans, including that caused by *S. pneumoniae*. In humans, *S. iniae* infection commonly causes soft tissue diseases such as cellulitis of the hand. This resembles soft tissue diseases including pharyngitis, necrotising faciitis and myositis caused by *S. pyogenes*. Thus the infection of zebra fish with *S. iniae* reproduces many features of many important streptococcal infections in humans, several of which can cause life-threatening systemic disease (Neely 2002).

S. pyogenes can also infect and cause disease in zebra fish. The pathogenesis of the infection is distinctly different from that of *S. iniae* and very similar to that reported for experimental, fatal intramuscular infection in primates. Use of the *S. pyogenes*-zebra fish model has identified *S. pyogenes* genes that contribute to pathogenicity in both fish and mammalian models. These include genes involved in toxin expression, peptidoglycan resistance to lysosyme and putative ABC transporter proteins (Neely 2002). This model has allowed the identification of genes important in the production of disease. This suggests that it will be of use for the investigation of other pathogenic species that are also capable of infecting fish.

1.5.2. Insects

The fruit fly *Drosophila melanogaster* spends a part of its life cycle in decaying organic matter, such as dead and rotting fruit. In this environment it often acts as a vector for microbial infection. The adult flies transmit yeast and bacteria from one plant to another while the larvae deliver them deeper into the fruit as they burrow (Tzou 2002). As a result *Drosophila* has evolved efficient mechanisms to prevent microbial infection.

In addition to the impermeable chitinous cuticle of the gut *Drosophila* also possesses a complex innate immune system that protects it from pathogenic attack. Initially disruption of the cuticle induces rapid proteolytic cascades that lead to blood clotting and melanisation. This allows the production of cytotoxic reactive oxygen species. Secondly a cellular immune response is initiated. This is mediated by different types of blood cells or haemocytes that participate in pathogen clearance by phagocytosis (Dushay and Eldon 1998). Finally, a set of inducible effector molecules are produced during systemic infection. These include antimicrobial peptides (AMPs), stress response proteins, factors involved in the opsonisation of invading organisms and iron sequestering molecules. Production of these factors occurs in an organ called the fat body which has been likened to the mammalian liver (Tzou 2002).

The signal transduction cascade that underlies this process has been the subject of intense investigations. They have revealed that there are numerous similarities to the mammalian innate immune response. In both insects and mammals, Toll family receptors signal through Rel family transactivators, mediating responses that are specific to different classes of pathogens (D'Argenio, Gallagher *et al.* 2001). Thus the genetic tractability of *D.*

melanogaster makes it an ideal model for the study of the mammalian innate immune response to bacterial pathogens.

D. melanogaster is susceptible to a wide range of human bacterial pathogens, including *P. aeruginosa*. When injected with *P. aeruginosa* strain PA01, bacteria grow exponentially within the fly, which eventually dies. This observation suggests that *D. melanogaster* could be used as an alternative model of infection that could be used screen mutants of *P. aeruginosa* for attenuated phenotypes.

Through the use of this model D'Argenio *et al* (D'Argenio, Gallagher *et al.* 2001) screened approximately 1500 transposon-insertion mutants of *P. aeruginosa* strain PA01 for those that were impaired in fly killing. The screen subsequently identified 33 mutants that displayed an attenuated phenotype when compared to the parent wildtype strain. All of those mutants that were strongly impaired in fly killing also had defects in twitching motility. However, twitching motility was not required for full virulence in *D. melanogaster*. This suggested that the genes required for twitching motility and fly killing control the expression of yet unknown virulence factors important in pathogenicity in fruit flies (Tan 2002). The *pil /chp* transduction system consisting of the *pil*GHIJKL *chp*ABCDE gene cluster in which all of the mutations occurred may be important for virulence in both flies and mammals (D'Argenio, Gallagher *et al.* 2001). Other insect species have also been used for the study of bacterial virulence such as the larvae of the greater wax moth *Galleria mellonella* and the larvae of the silk moth *Bombyx mori*. These are susceptible to a range of opportunistic human bacterial pathogens similar to *D. mellanogaster*. This includes *Proteus vulgaris*, *Serratia marcecsens* and *P. aeruginosa* but importantly also *V. cholerae* and *S. aureus* (Jander, Rahme *et al.* 2000; Kaito 2002).

B. mori the silk worm although susceptible to infection by *P. aeruginosa* strain PA14 is also colonised and killed by strains of *S. aureus* , *V. cholerae* and EHEC 0157. Again mutants with reduced virulence in the silk moth also exhibit reduced virulence in mammalian models of infection. Additionally it has also been shown that these infections can be eradicated through the use of clinically relevant antibiotics. This suggests that supplementary to the investigation of the molecular events of the host-pathogen interaction, *B. mori* may also be useful for the preliminary evaluation of antibiotics prior to their investigation in mammalian models (Kaito 2002).

Other attributes of *B. mori* that make it particularly attractive as an alternative animal model of bacterial infection is its relatively large mass of 150-250 mg. This enables the injection of defined doses of bacteria that allows the examination of the pathology of the infection and the calculation of the precise dose at which 50 % of animals die (LD_{50}) something not easily attainable with other alternative models (Jander, Rahme *et al.* 2000).

1.5.3. Amoeba

Dictyostelium discoideum is a bactivorous, unicellular amoeba that lives in soil. Usually the cells exist in the amoebal form and are highly motile, ingesting bacterial cells through phagocytosis. However, during starvation, the organism undergoes a complex developmental cycle in which the normally free-living cells aggregate to form a multicellular organism. This takes the form of a motile, phototactic slug that further develops into a fruiting body containing *D. discoideum* spores. These are then released to colonise other areas where nutrients are available after germination again to the amoebal form (Solomon, Rupper *et al.* 2000).

The endosomal and phagosomal processes involved in *D. discoideum* phagocytosis have been well characterised and found to be very similar to that of mammalian macrophages (Solomon, Rupper *et al.* 2000). This has lead to the use of *D. discoideum* as a model for the study of the host-pathogen interactions between mammalian macrophages and intracellular bacterial pathogens.

Usually, the study of macrophage-pathogen interactions is hampered by the fact that these cells are not amenable to genetic analysis. However the availability of suitable genetic tools makes *D. discoideum* a genetically tractable host. The organism is haploid and has a relatively small genome of 34 Mb. It is possible to transform cells through electroporation and inactivate genes by homologus recombination and marker replacement. There are also plasmids that replicate in the amoeba that can be used for complementation or ectopic expression of artificially introduced genes. This along with the availability of a complete genome sequence makes *D. discoideum* a particularly powerful model for the study of pathogen interactions with macrophages (Solomon, Rupper *et al.* 2000; Pukatzki 2002; Tan 2002).

The *D. discoideum* model has been used to study the interactions between *L. pneumophila* and human macrophages. The pathogenesis of the bacterium within mammalian hosts and its ability to grow in *D. discoideum* has been shown to be closely linked. There is also strong genetic evidence that the molecular basis for *L. pneumophila* invasion of amoeba and entry into human macrophages are similar (Tan 2002).

Through the use of this model it was found that growth of *L. pneumophila* in *D. discoideum* is dependant on functions provided by so called *dot/icm* genes that are responsible for contact-dependant cytotoxicity. These may form a transport system responsible for transferring an as yet unidentified effector protein or proteins in to the host

cell, causing lysis and death (Solomon, Rupper *et al.* 2000). The relevance of this model to mammalian pathogenesis is further supported by the finding that mutants in each of the *dotH/icmK, dotI/icmL* and *dotO/icmB* genes that had severe intracellular growth defects in mouse bone marrow-derived macrophages also failed to grow intracellularly in *D. discoideum*.

The alternative animal models of bacterial infection already described have all been shown to be effective for the investigation of bacterial pathogenicity. They allow the investigation of the various factors that contribute to pathogenesis in the mammalian host at the molecular level, while only utilising a minimum of higher order organisms to confirm these observations. However, although these models show some utility the most widely used and best-characterised pathogenesis model, currently in use in many laboratories is the nematode worm *Caenorhabditis elegans*.

1.5.4. The nematode *Caenorhabditis elegans*.

C. elegans is a free-living nematode round-worm belonging to the family Rhabditidae in the phylum Nematoda. Members of this phylum are many and diverse and have become well adapted to a free-living existence in nearly all terrestrial and marine habitats. Some have also adopted a parasitic mode of life in a wide variety of plant and animal hosts (Wixton, Blaxter *et al.* 2000).

C. elegans is found in many parts of the world mainly among decaying vegetation where it lives by grazing on microbes (Wixton, Blaxter *et al.* 2000). The female is a self-fertilising protandrous hermaphrodite, which first produces sperm and then oocytes forming a brood of about 300 eggs. The male although a similar size, at approximately 1mm, is morphologically distinct and only occurs at a very low frequency in the population. The male worm may fertilise the hermaphrodite, but hermaphrodites are not able to fertilise each other (Wood 1988).

C. elegans is able to propel itself forward or backward through co-ordinated undulatory movements of the body wall muscles. It may respond to a variety of stimuli such as touch, temperature and many different volatile chemical compounds. When placed on a temperature gradient they tend to move towards the temperature to which they were previously exposed through a process called nociception (Wittenburg 1999).

Juvenile worms hatch from the egg and develop through four larval stages, punctuated by a moulting of the cuticle, to allow further maturation to the next larval stage. If conditions become unfavourable during larval development an alternative to the usual third larval

stage may be produced, called the dauer larvae, which is resistant to a variety of environmental stresses. When conditions improve the dauer moults and continues its development. The emerging adult is fertile for a period of approximately 4 days during which time egg laying occurs along with mating if male worms are present. The adult then lives for further 10-15 days after which it dies (Wood 1988).

C. elegans can be easily maintained in the laboratory where it can be grown on agar plates seeded with *E. coli.* It may also be cultured in a liquid media either with *E. coli* or axenically to a high population density. Throughout its life cycle the body is transparent allowing observation of cellular differentiation at each developmental stage through the use of differential interference contrast (DIC) Normarski imaging techniques (Schnabel 1999). In this way the entire developmental history of every cell in the nematode, from the founder cells of the early events of embryogenesis within the egg to adulthood has been described in detail. The application of electron microscopy to serial sections of the nematode has also allowed the construction of a list of the identified epithelial, muscle, neuronal and other cell types that comprise the entire anatomy of the adult nematode (Hodgkin, Palsterk *et al.* 1995).

C. elegans is not only simple biologically but also relatively simple at the genetic level. The haploid set of chromosomes includes five autosomes and a sex chromosome which are all approximately equal in size, giving a total of approximately 8×10^7 bp, which apparently approaches the limit for a differentiated metazoan animal (Hodgkin, Palsterk *et al.* 1995). Sex is determined chromosomally, depending on the ratio of autosomes to sex chromosomes. Hermaphrodites are diploid for all six chromosomes (XX), whereas males are diploid for the autosomes only and have one X chromosome (XO). Males arise

spontaneously in populations by X-chromosome non-disjunction at meiosis, with a frequency of approximately 1 in 500 individuals (Wood 1988).

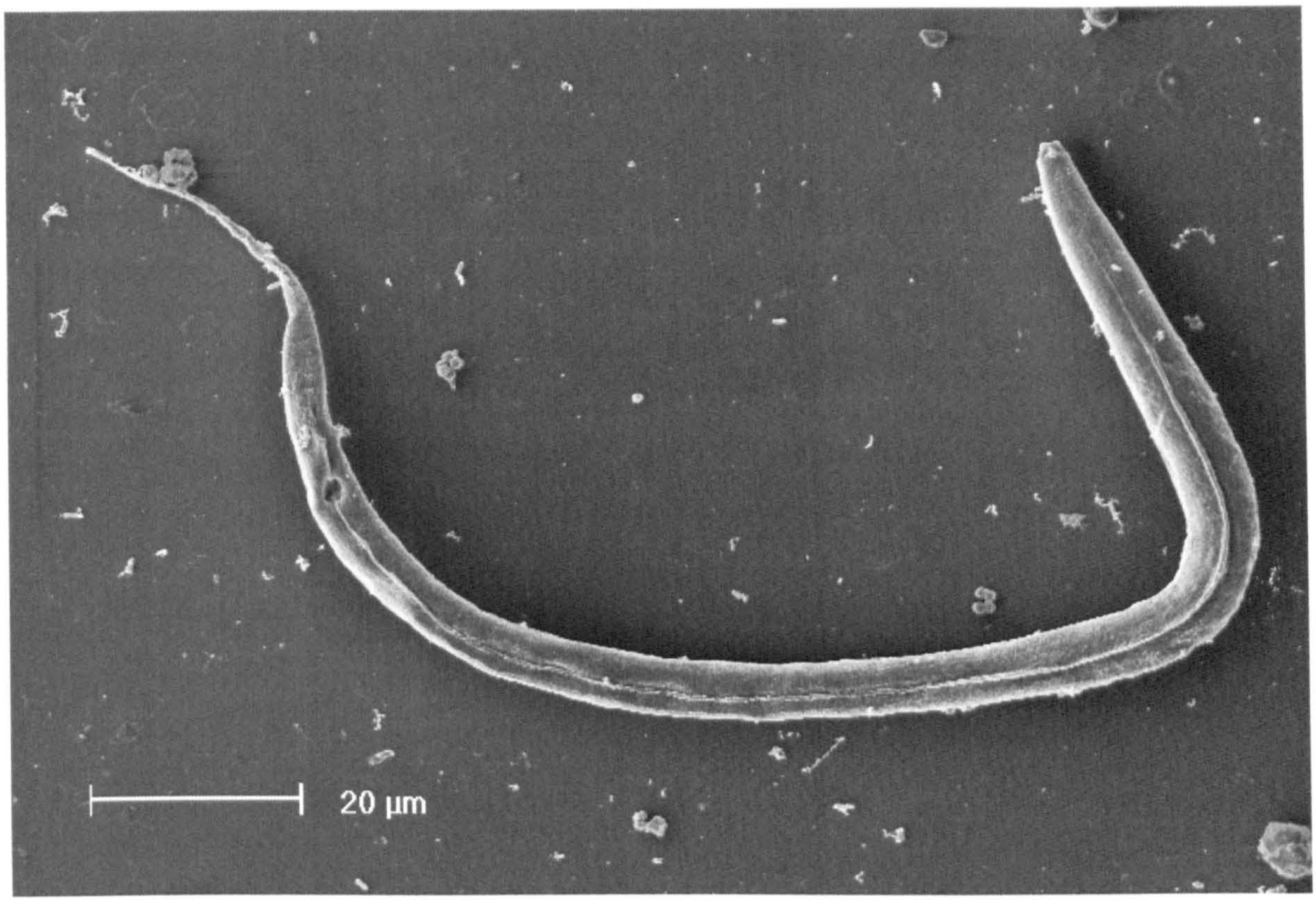


Figure: 1.1. Scanning electron micrograph of the nematode *C. elegans*. The size of the specimen indicates that it is in the second larval stage. Specimens were prepared using the method described in section: 2.2.2.2.

1.5.4.1. The *C. elegans* life history.

The nematode life history starts with the fertilisation of mature oocytes by the hermaphrodites own sperm or sperm obtained from a male at a previous mating. This is stored within a specialised storage organ called the spermatheca until required for fertilisation. During the first 30 minutes after fertilisation the chitinous eggshell develops, derived from components within the egg. This tough layer renders the egg impermeable to most solutes and allows survival outside the uterus (Wood 1988). The chitinous layer is often the thickest layer comprising the eggshell and gives structural strength to protect the developing embryo within (Wharton 1980).

The eggs are retained for approximately 3 hours and are then deposited through the vulva to the outside environment. Embryogenesis follows where selected cells move, proliferate and die according to a precisely timed pattern that is identical from one growing embryo to another. This gives rise to a fixed number of cells with pre-determined fates. Approximately 7 hours after fertilisation cell proliferation ceases almost entirely and during the following 7 hours the developing body elongates, neuronal processes grow and the cuticle is secreted (Wood 1988). A photomicrograph of the *C. elegans* egg is shown in Figure 1.2. a.

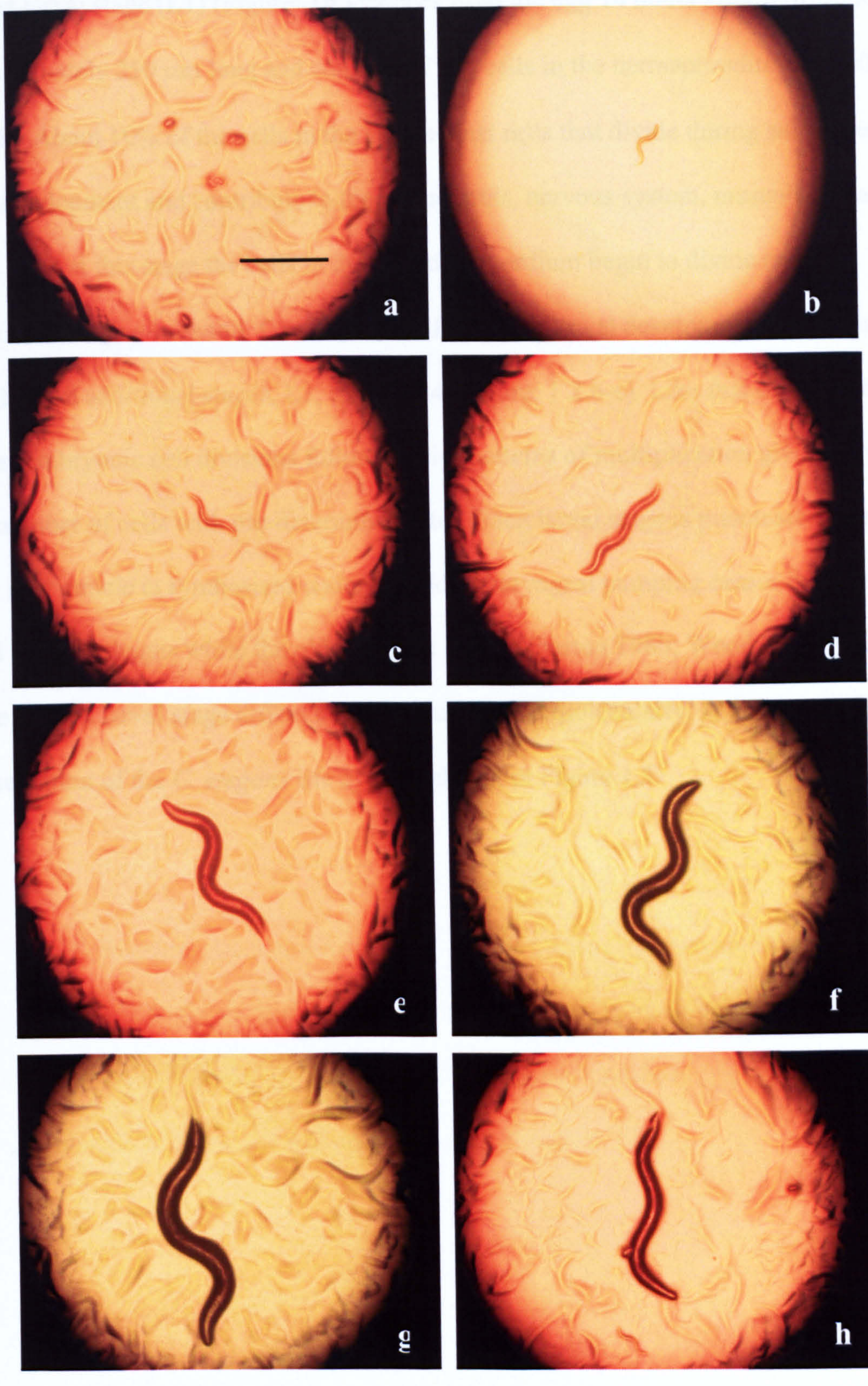


Figure: 1. 2. The developmental stages of the nematode *C. elegans*. Figure 1a: eggs, 1b-1e: hermaphrodites from L1 to L4 stage respectively. 1f: a young adult, 1g: fully-grown adult hermaphrodite and 1h: adult male. Scale bar = 400 μm.

The first larval stage (L1) (Figure: 1b.) hatches from the egg 14 hours after fertilisation. It is approximately 250 μm long and consists of 558 cells in the hermaphrodite and 560 in the male. About 10% of the cells in the L1 are blast cells that divide during subsequent larval development and contribute to the hypodermis, nervous system, musculature and gonad. Also at this stage the cells in the gonad primordium begin to divide and this continues through adulthood. Over a period of 50 hours larval development proceeds through three additional stages, L2, L3 and L4 (Figure: 1.2.b-e). Like that of the embryo, the post embryonic cell divisions follow precise patterns of multiplication giving rise to a fixed number of cells with pre-determined fates. Each larval stage is punctuated by a moult where new cuticle is synthesised under the old. Pharyngeal pumping ceases during this period called lethagus while the old cuticle is shed (Wharton 1980; Wood 1988). The cuticle at each larval stage differs both structurally and molecularly. A diagrammatic representation of the *C. elegans* life cycle is shown in Figure 1.3.

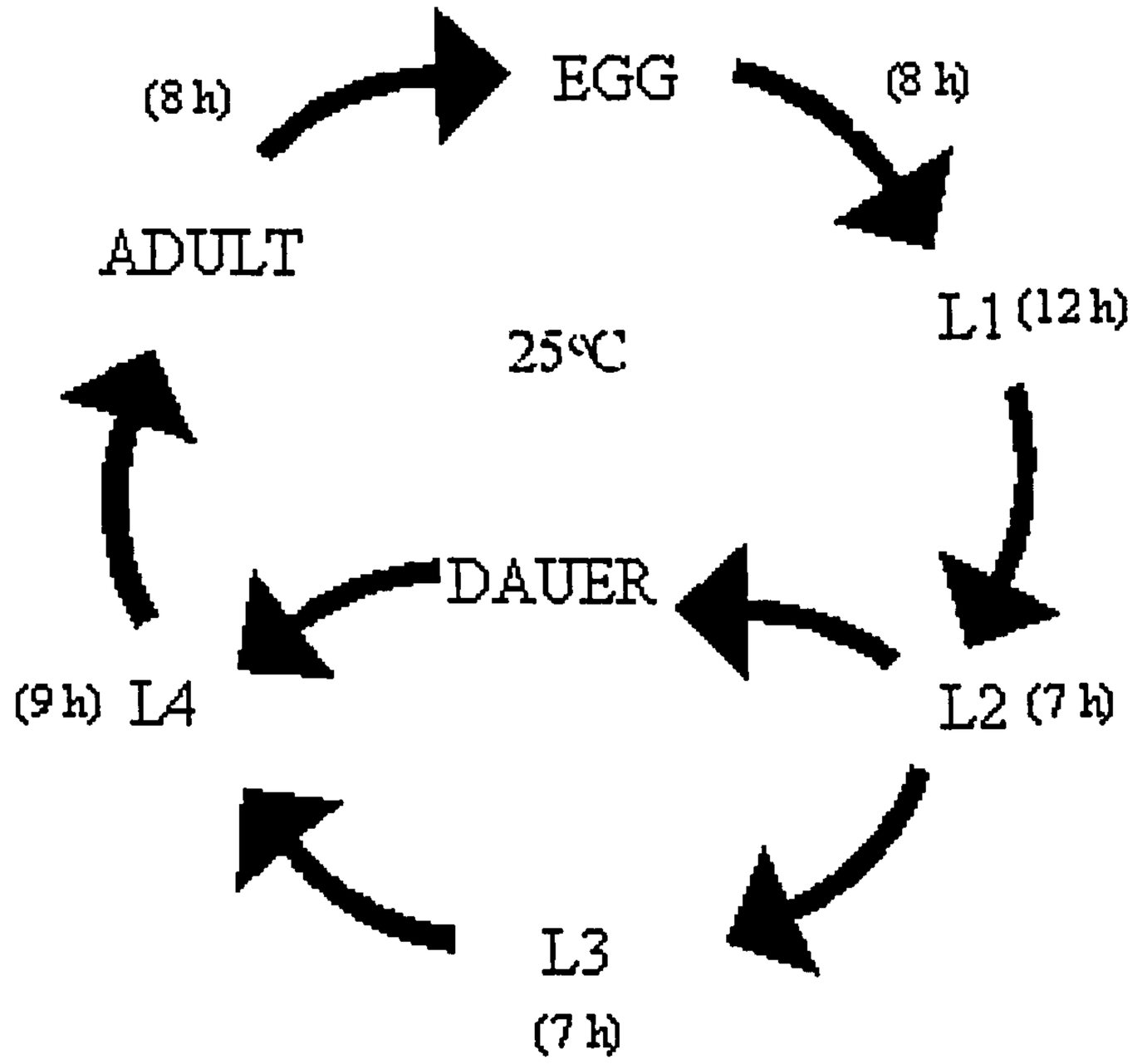


Figure: 1.3. A diagrammatic representation of the life cycle of the nematode *C. elegans*. The duration of each larval stage is shown in parentheses. The black arrows represent moults. The 8 hours from the egg to L1 stage is the interval between egg laying and hatching. The entire life cycle takes approximately 3 days at 25°C.

1.5.4.2. The Dauer Larvae.

The dauer larvae (German; enduring) (Figure: 1.4.) is an alternative third larval stage that is formed under conditions of environmental stress. Environmental signals such as lack of food or adverse temperature promote the formation of the characteristic stage in *C. elegans*, which is adapted for long term survival and dispersal (Riddle 1988). The concentration of a specialised dauer pheromone, produced constitutively by the population, acts as a measure of population density and also prompts the transition to the dauer stage under conditions of over-crowding. The dauer is non-feeding and has morphological adaptations that allow it to become resistant to a range of stresses including dessiccation, chemical detergents and temperature. The larvae show behaviour that is not seen in any other larval stage. Feeding and pharyngeal pumping is completely stopped and they often lie motionless. However they show a rapid response to touch by moving away from the stimulus. Dauer larvae also have a tendency to climb up objects that protrude from the substrate. They have the unique ability to stand on their tails and wave their heads back and fourth. This may be an adaptation to aid in the dispersal of the organism through attachment to insects or larger animals (Cassada and Russel 1975).

The dauer larvae are considered to be non-ageing because the duration of the dauer diapause does not affect the post-dauer life span (Riddle 1988). They may be maintained for up to 70 days (Klass and Hirsh 1976). If they are subsequently placed under more favourable conditions, developmental commitment to exit the dauer stage occurs within 1 hour. After approximately 2-3 hours the larvae begins to feed and resumes its development by moulting to the L4 stage (Riddle 1988).

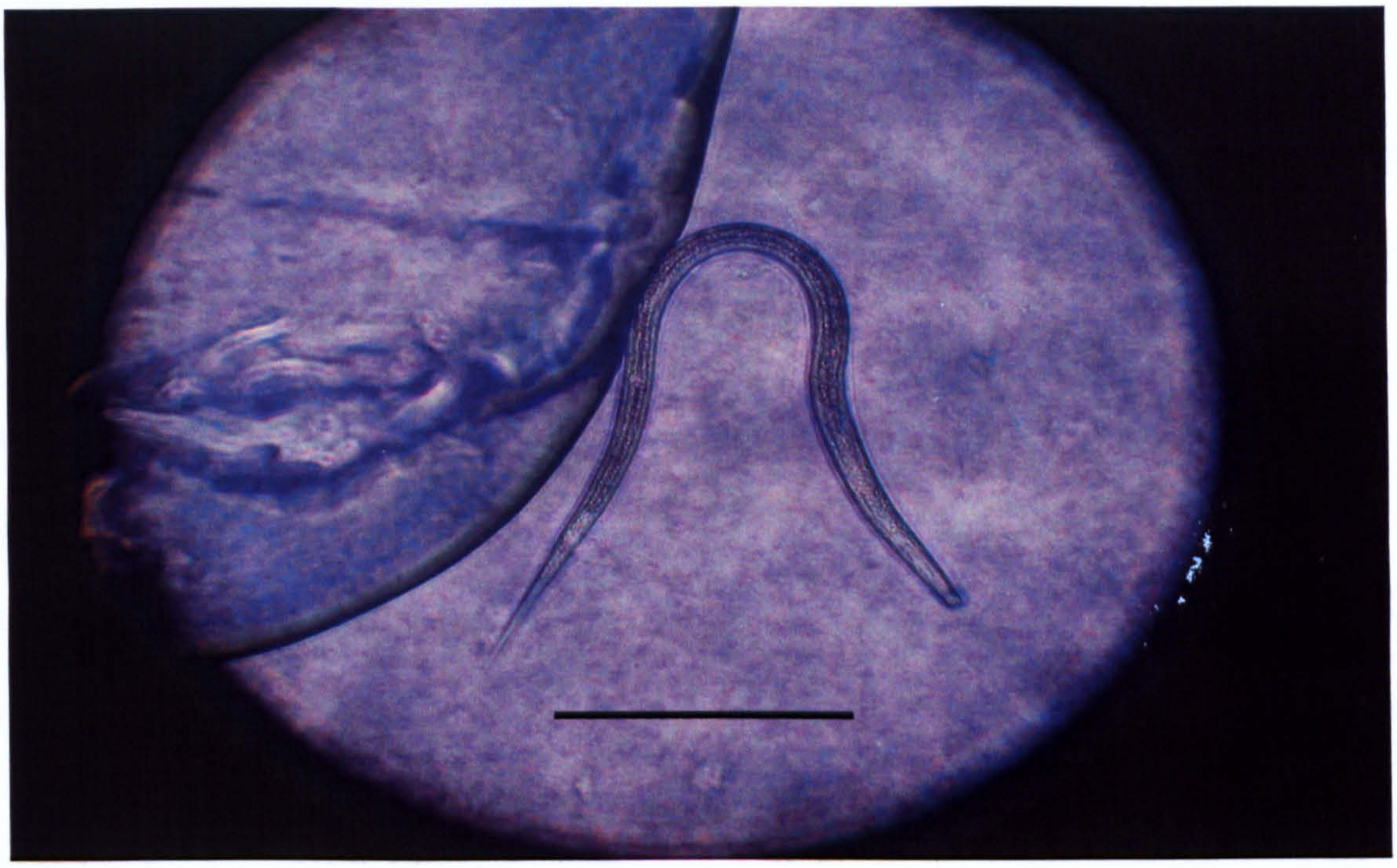


Figure: 1.4. The non-ageing dauer larval stage. Note the changes in morphology as compared to Figure: 1.2.d a normal L3 larvae. The dauer L3 larvae is longer and thinner than the normal L3 larvae with a dark colouration due to storage granules within the gut. Scale bar represents 200 μm.

1.5.4.3. The L4 Larvae

At the fourth larval stage the formation of the gonad is complete. In the hermaphrodite, sperm production begins which is stored in the spermatheca. At the final moult to adulthood the sperm production stops and subsequent meiosis and differentiation generates only oocytes. A characteristic and diagnostic feature of the fourth larval stage is the vulval plug. This consists of a single cell that blocks the opening of the vulva to the outside environment. At the final moult this is lost with the shed cuticle opening the vulva and allowing eggs to be laid. Figure: 1.5. shows the vulval plug as a white spot plugging the vulva. This allows easy differentiation of the L4 larvae from the other larval stages by microscopy (Wood 1988).

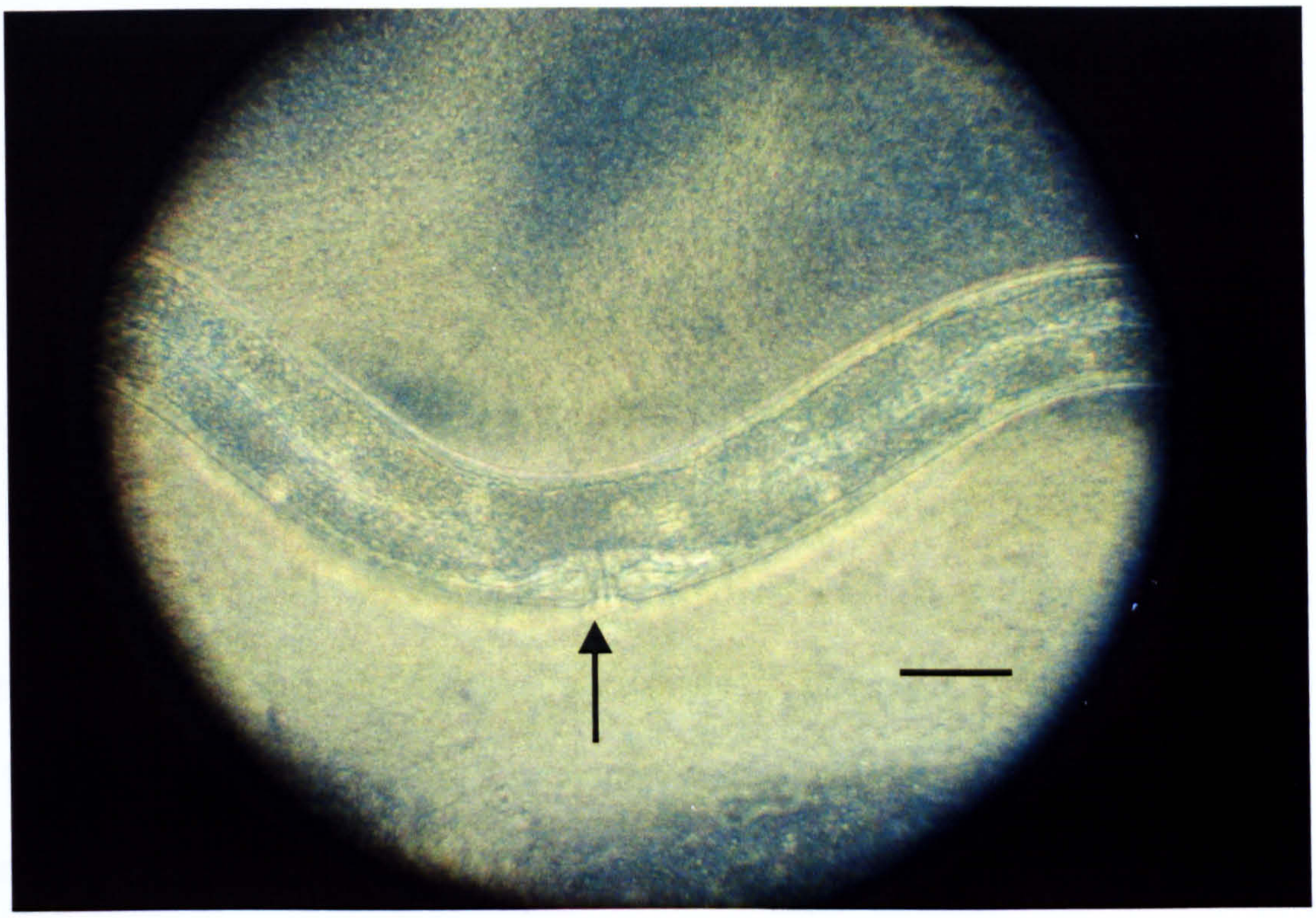


Figure: 1.5. A phase contrast photomicrograph of the vulva of an L4 larva. The arrow shows the vulval plug that is characteristic of the L4 larval stage. Scale bar represents 20 μm.

1.5.4.4. The Adult Male

Males are morphologically distinct from hermaphrodites as they are smaller and have specialised structures for mating. They also have a number other less obvious differences involving behaviour, musculature and the nervous system (Hodgkin 1988). The development of the male gonad follows a different lineage as it is geared primarily to the production of sperm only and not oocytes as in the hermaphrodite. Other physiological differences include the intestine, as in the hermaphrodite this is the site of yolk production. The region of the biggest physiological difference is the tail. The tail contains several complicated sex specific structures. These include the phasmids, sensory appendages that are involved in the positioning of the copulatory apparatus during mating. The tail is comprised of an acellular cuticular fan supported by rays. There are two copulatory spicules that lie in channels associated with the genital tract. These are extensively sclerotized giving them structural rigidity. During mating these are inserted into the vulva of the hermaphrodite by means of the protractor muscles, muscles in the tail region specialised for copulation. Sperm is introduced which is stored in the spermatheca and preferentially fertilises the oocytes (Hodgkin 1988). Males are capable of cross-fertilising hermaphrodites and yield equal numbers of males and hermaphrodites. However the higher growth rate of the hermaphrodite population means the frequency of males in the population remains low. Males have a longer reproductive life than that of the hermaphrodite and may produce up to 3000-4000 functional sperm during the 6 days of their fertility (Hodgkin 1988).

1.5.4.5. Innate immunity in *C. elegans.*

The term innate immunity refers to a number of evolutionarily ancient mechanisms that are used by plants and animals to defend themselves against infection (Mallo 2002). Although a very simple organism, *C. elegans* has a complex innate immune system, which shows similarities to that of insects and mammalian species. This is comprised of a set of inducible lipases, lysosymes and lectin-binding proteins under the control of the transforming growth factor β (TGF-β)-related gene *dbl*-1. These are expressed in the intestinal tract of the nematode and act directly against invading microorganisms and may serve in self, non-self recognition of pathogens as in other organisms (Mallo 2002).

The *C. elegans* innate immune system also contains a pathway with homologies to the mammalian p38 mitogen activated protein kinase (MAPK) pathway. The p38 homologues PMK-1 and PMK-2 signal through SEK-1 and NSY-1. These are orthologues of the mammalian MAPK kinase MKK3/MKK6 and MAPK kinase kinase ASK-1 respectively. This mechanism has been shown to mediate programmed cell death in the nematode germ line in response to *S. enterica* serovar Typhimurium infection. Mutations in the corresponding genes also give rise to an enhanced susceptibility to infection (Esp) phenotype indicating their importance in the *C. elegans* innate immune system (Kim 2002; Aballay 2003).

Finally homologues of mammalain Toll-like receptors (TLR) have been found in *C. elegans.* These encode transmembrane proteins containing extracellular leucine-rich repeats (LRR) and an intracellular domain known as the Toll/interlukin receptor (TIR) domain. In mammals the LRR domains of TLR's are involved in the recognition of

conserved molecular patterns of pathogens, activating the innate immune response (Kopp 1999; Tan 2002). However, unlike the Toll systems found in humans and fruit flies *C. elegans* only possesses one Toll-homologue, *tol*-1. This is only expressed in cells of developing larvae and in certain classes of neurones in the adult nematode. Studies have shown that instead of functioning to arm pathways of the innate immune system as in other organisms it is essential for correct larval development. It has also been shown to function in pathogen recognition but due to its neuronal localisation it modulates behaviour to avoid potential pathogens (Pujol, Link *et al.* 2001).

1.5.4.6. *C. elegans* as an alternative model of bacterial infection.

In 1965 Sidney Brenner first proposed the use of *C. elegans* as promising model organism for a concerted genetic, ultrastuctural and behavioural investigation of development and function of a simple nervous system (Wood 1988). As a result of this vision *C. elegans* became the first and so far only animal for which the complete cell lineage and neuronal wiring diagram is known. As a further consequence of Brenner's original work, in 1998, *C. elegans* provided the first complete genome sequence to be determined for any multicellular organism (Hodgkin 2002). Since this time *C. elegans* has been used as the typical model metazoan and has allowed the investigation of cell lineage and function in other free-living and parasitic nematode species. The availability of a complete *C. elegans* genome sequence has also allowed the deduction of the structure and function of genes in other nematode species due to the large amount of structural homology seen between genes of members of this phylum (Wood 1988). In addition to its role as a model organism for biological processes in eukaryotes, recent studies have shown that useful attributes of the nematode model may exist in the investigation of host-pathogen interactions.

A variety of both Gram-negative and Gram-positive bacterial pathogens have been shown to cause the death of *C. elegans* by three different mechanisms. These correspond to infection of the intestinal tract, intoxication from bacterial exoproducts and blockage of the alimentary tract. Known pathogens of *C. elegans* and their killing mechanism are summarised in Table: 1. This table includes opportunistic pathogens such as *P. aeruginosa* and *S. marcsescens* and human pathogens such as *S. enterica* serovar Typhimurium. For those pathogens, the *C. elegans* model has been used to investigate the host–pathogen interactions that occur during infection. This has allowed the identification of novel genes essential for pathogenesis in nematodes. Further work has also shown that some of these genes also play a role in the pathogenesis of disease in mammals and has provided valuable information regarding the pathogenesis of these organisms.

Table: 1.1. Known bacterial pathogens of the nematode *C. elegans* and killing mechanisms, which they use to cause death of the nematode in culture. * Does not cause significant disease in humans.

Species	Killing mechanism	Reference
Gram Negative		
P. aeruginosa	Intoxication/ Infection	(Darby, Cosma *et al.* 1999; Miklos, Tan *et al.* 1999)
*P. fluorescens**	Intoxication/ Infection	(Tan, Miklos *et al.* 1999)
B. cepacia	Intoxication/ Infection	(O' Quinn, Wiegand *et al.* 2001; Tan 2002)
B. pseudomallei	Intoxication/ Infection	(O' Quinn, Wiegand *et al.* 2001)
*Burkholderia thailandensis**	Intoxication/ Infection	(Gan 2002)
Serratia marcescens	Intoxication/ Infection	(Aballay and Ausubel 2002)
S. enterica serovar Typhimurium	Infection	(Aballay, Yorgey *et al.* 2000; Labrousse, Chauvet *et al.* 2000)
Y. pestis	Blockage	(Darby 2002)
Yersinia pseudotuberculosis	Blockage	(Darby 2002)
Aeromonas hydrophilia	Infection	(Ewbank 2002)
*Agrobacterium tumefaciens**	Infection	(Ewbank 2002)
Erwina chritamthemi	Infection	(Ewbank 2002)
*E. carotovoa carotovora**	Infection	(Ewbank 2002)
Shewanella massalia	Infection	(Ewbank 2002)

Shwanella putriefaciens	Infection	(Ewbank 2002)
Gram -positive		
*Bacillus megaterium**	Intoxication	(Andrew and Nicholas 1976)
*Bacillus thuringiensis**	Intoxication	(Leyns 1995; Couillault 2002)
Enterococus faecalis	Infection	(Garsin, Sifri *et al.* 2001)
*Microbacterium nematophilum**	Infection	(Hodgkin, Kuwabara *et al.* 2000)
S. aureus	Infection	(Sifri 2003)
S. pneumoniae	Intoxication	(Jansen 2002)

Despite the evolutionary distance between *C. elegans* and that of the higher order mammalian models, there exists a large degree of homology between the genomes of these organisms. For example 36% of the 19.000 predicted proteins of the *C. elegans* genome have matches in humans (Darby, Cosma *et al.* 1999). This allows it to be used in the correct context to identify new genes of related function and elucidate pathways within which such genes act. The *C. elegans* pathogenesis model then allows host-pathogen interactions at the molecular level to be dissected. The genetic tractability of both host and the infecting organism along with the availability of standard genetic techniques has greatly increased the ease with which the molecular basis of pathogenicity can be investigated. The use of non-mammalian hosts has also greatly facilitated our understanding of the molecular basis of the universal virulence mechanisms conserved throughout evolution. These underlie the host-pathogen interactions seen in many of the organisms investigated and allow them to infect a broad range of hosts from taxonomically distant phyla. As infection of *C. elegans* has been shown to be directly relevant to the pathogenesis of disease in mammalian hosts, it may also provide a valuable insight into the processes that lead to disease in higher-order mammalian species such as humans.

1.6. Aims.

In this study, techniques for the investigation of bacterial pathogenicity using the nematode *C. elegans* were assessed. Novel applications for existing techniques using nematodes were explored and used to establish a *C. elegans* model of infection. The validity of the nematode as a model of *B. pseudomallei* infection was then assessed through investigation of a range of *Burkholderia* strains. The established *C. elegans-Burkholderia* model was then used to study the pathogenesis of *B. pseudomallei* at the cellular and molecular level through the use of TEM, the investigation of *B. pseudomallei* mutants and the study of genomic cosmid libraries. Finally the usefulness of *C. elegans* as an alternative model of *B. pseudomallei* infection was discussed.

Chapter: 2. Materials and methods.

2.1: Microbiological Methods.

2.1.1: Preparation of media.

2.1.1.1. Nematode growth media (NGM).

Unless otherwise indicated growth of *C. elegans* and challenge experiments involving nematodes took place on this media. The constituents were as follows.

Sodium chloride	3 g/l
Noble agar	17 g/l
Peptone	2.5 g/l

Made up to 1l with distilled water.

The mixture was sterilised by autoclaving at 121 °C for 15 minutes.

After cooling to 55 .C a number of supplementary additives were added to facilitate the growth of the bacterial strains used.

Amounts are per litre of molten, sterile NGM agar.

1M Potassium phosphate Buffer (pH 6)	25 ml/l
1M Calcium chloride solution	1 ml/l
Uracil 2 mg/ml	1 ml/l
1M Magnesium sulphate solution	1 ml/l
Cholesterol in ethanol 10 mg/ml	0.5 ml/l

Due to its heat labile nature uracil was sterilised using a 0.22 . m filter prior to use.

2.1.1.2. Luria-Bertani (L B) Media.

Unless otherwise stated all bacterial strains were initially cultured in LB media.

Difco Bacto tryptone	10 g/l
Difco bacto yeast extract	5 g/l
Sodium chloride	5 g/l
Difco bacto agar (plates only)	20 g/l

The ingredients were dissolved in distilled water and made up to the appropriate volume before sterilising by autoclaving at 121 °C for 15 minutes.

2.1.1.3. Freezing Solution.

This buffered solution was used in the preparation of frozen nematode stocks.

1M Sodium chloride	100 ml/l
1M Potassium phosphate buffer at pH 6.0	50 ml/l
Glycerol	300 ml/l

The constituents were added to 1l of distilled water and sterilised by autoclaving at121 °C for 15 minutes. Prior to use 150 . l/l of sterile 1M $MgSO_4$ was also added after autoclaving.

2.1.1.4. K-Medium.

K- medium was used as a buffer for the removal of nematodes from plates and the re-suspension of bacteria when necessary.

Sodium chloride	3.075 g/l
Potassium chloride	2.42 g/l

Added to 1l of distilled water and sterilised by autoclaving at 121 °C for 15 minutes.

2.1.1.5. SOC Broth.

SOC broth was used as a recovery medium for transformed bacterial cells.

Bactotryptone	2 g
Bacto yeast extract	0.5 g
Sodium chloride	10 mM
Potassium chloride	2.5 mM
Magnesium sulphoxide	10 mM
Glucose	20 mM

Dissolve in 100ml of dH_2O and autoclave at 121 °C for 15 minutes. Store at -20 °C until needed.

2.1.2: Bacterial Strains.

Bacterial strains used in this study are listed in Table: 2.1. *E. coli* OP50 was routinely cultured as described by Sambrook *et al* (Sambrook, Fritsch *et al.* 1989). *E. coli* OP50 has a genetic lesion that prevents the *de novo* synthesis of uracil, which must be added growth media to a final concentration of 0.2 . g/ml. *E. coli* OP50 was cultured on NGM to provide a food source for the nematodes. *P. aeruginosa* strains, *B. pseudomallei* and *B. cepacia* complex strains were routinely cultured in LB broth overnight unless otherwise stated.

All bacterial pathogens described were cultured under appropriate containment conditions, in sealed flasks shaking at 200 rpm. Bacteria were harvested by centrifugation at 3000 x g for 15 minutes and resuspended in K-medium before use in nematode feeding inhibition experiments.

Table: 2.1. Bacterial strains used in this study. Synonymous strain numbers are shown in parentheses. Genotypes where applicable are shown below the strain name.[1]. National collection of type cultures (NCTC) Central Public Health Laboratory, 61 Colindale Avenue, London. http://www.hpa.org.uk/srmd/div_cdmssd_nctc/index.htm, [2]. American Type Culture Collection (ATCC) http://www.atcc.org, [3]. National Collection of Industrial and Marine Bacteria Ltd (NICMB) http://www.ncimb.co.uk, [4]. Belgium Coordinated Collections of Microorganisms/ Laboratorium Microbiologie Ghent (BCCM/LMG) http://www.belspo.be/bccm,[T]. Type strain for that genomovar.

Species and Strain	Source or Reference
E. coli OP50	*Caenorhabditis* genetics centre, University of Minnesota, USA (Brenner 1974)
P. aeruginosa PA14	(Rahme, Stevens *et al.* 1995)
P. aeruginosa PA01	(Holloway 1955)
B. pseudomallei	
B. pseudomallei 576	Dr. David Dance, Health Protection Agency, Plymouth
B. pseudomallei K96243	
B. pseudomallei NCTC 4845	NCTC [1] (Mack 1996)
B. pseudomallei 204	Dtsl Porton Down
B. pseudomallei BRI	Dstl Porton Down
B. pseudomallei ATCC 23344	ATCC [2]
B. pseudomallei 52	Dr. T. L. Pitt. Central Public Health Laboratory, London.
B. pseudomallei 2889	Dr. T. L. Pitt. Central Public Health Laboratory, London.
B. pseudomallei 42	Dr. T. L. Pitt. Central Public Health Laboratory, London.
B. pseudomallei Δ*pil*A	Dr. A. Hayes, Dstl. Porton Down.

B. pseudomallei 6H2	This study
Transposon Mutant Strains of *B. pseudomallei*	Dr. T. Atkins. Dstl., Porton Down (Atkins 2002)
B. thailandensis	
B. thailandensis E254	DSTL Porton Down
B. thailandensis E135	DSTL Porton Down
B. thailandensis E125	DSTL Porton Down
B. thailandensis E132	DSTL Porton Down
***B. cepacia* Complex** (Mahenthiralingam, Coenye *et al.* 2000)	
Genomovar I	
B. cepacia ATCC 25416^{T}	ATCC
B. cepacia ATCC 17759	ATCC
B. cepacia J2540	Dr. J. Govan, University of Edinburgh.
B. cepacia C2970	Dr. J. Govan, University of Edinburgh.
B. cepacia C1963	Dr. J. Govan, University of Edinburgh.
B. cepacia C1964	Dr. J. Govan, University of Edinburgh.

B. cepacia C3159	Dr. J. Govan, University of Edinburgh.
B. cepacia LMG 6860 (NCIMB 9091)	NCIMB [3]
Genomovar II	
B. multivorans LMG 13010 [T]	BCCM/LMG [4]
B. multivorans ATCC 17616	ATCC
Genomovar III	
B. cenocepacia sp. nov. LMG 16656 [T] (J2315)	BCCM/LMG
B. cenocepacia sp. nov. LMG 18863 (K56-2)	BCCM/LMG
B. cenocepacia sp. nov. LMG 16654 (J415)	BCCM/LMG
B. cenocepacia sp. nov. C1394	Dr. J. Govan, University of Edinburgh.
B. cenocepacia sp. nov. J2956	Dr. J. Govan, University of Edinburgh.
B. cenocepacia sp. nov. C2836	Dr. J. Govan, University of Edinburgh.

Genomovar IV	
B. stabilis LMG 14294 ^{T}	BCCM/LMG
B. stabilis LMG 14086	BCCM/LMG
Genomovar V	
B. vietnamienesis LMG 10929 ^{T}	BCCM/LMG
B. vietnamiensis LMG 18836	BCCM/LMG
Transformation competent bacterial strains	
XL1 Blue MR *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F'*proAB lacI*^{q}*ZΔM15 Tn10* (Tet^{r})]	Stratagene, Amsterdam The Netherlands
XL10 Gold Tet^{r} *Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lacI*^{q}*ZΔM15 Tn10* (Tet^{r}) Amy Cam^{r}]^{a}	Stratagene, Amsterdam The Netherlands

2.2. Nematological Methods.

2.2.1. Culture of *C. elegans* wild type and mutant strains.

2.2.1.1. Culture on Solid Media.

C. elegans wild type N2 (var. Bristol) was obtained from the *Caenorhabditis* genetics centre (GCG, 250 Biological Sciences Centre, University of Minnesota, 1445 Gortner Avenue, St. Paul, MN, USA). Stocks were maintained by transferring 1 cm^2 pieces of NGM agar on which nematodes had already been cultured to fresh plates of NGM which had been previously seeded with *E. coli* OP50. Routine culture of *C. elegans* was at 16 °C for 4 days. *C. elegans phm-2* (Avery 1993) and *srf* mutant strains were also obtained from the CGC. Culture conditions are as described above for *C. elegans* N2.

2.2.1.2. Culture in Liquid Media.

A bacterial food source was first created through inoculation of a 100 ml of LB-broth with *E. coli* OP50 and incubating overnight at 37 °C. The resulting bacterial suspension was then centrifuged at 3000 x g for 15 minutes. The supernatant was discarded and the bacterial pellet resuspended in 10 ml of K-medium. The suspension was stored at 4 °C until required.

One 9 cm NGM plate of *C. elegans* was washed with 6 ml of K-medium and 5 ml containing the nematodes removed. The worm suspension was added to approximately 85 ml of K-medium along with 1 ml of the previously prepared *E. coli* OP50. The culture was

then placed at 16 °C on a rotating platform for 7 days. During this period the turbidity of the culture was monitored and if clearing occurred more bacterial suspension was added.

2.2.1.3. Preparation of frozen nematode stocks.

Nematode worms were washed from one 9 cm NGM plate with 3 ml of K-medium and centrifuged at 1000 x g for 5 minutes. After centrifugation 1.5 ml of the supernatant was removed and 1.5 ml of freezing solution added. 1 ml aliquots were placed in Nunc cryo-tubes (Nunc, UK). Prior to freezing the tubes were insulated with polystyrene packing to ensure a gradual freezing of the samples and placed at ¯70 °C. After 24 hours one of the tubes was thawed gradually. The bottom 250 . 1 containing the nematode worms was pipetted onto a NGM plate previously seeded with *E. coli* OP50 and incubated at 16 °C overnight. The plate was then examined using light microscopy at x 40 magnification using an inverted microscope to assess the approximate viability of the thawed stock. The remaining two tubes were removed from the polystyrene packing to a place of permanent storage at ¯70 °C.

2.2.1.4. Synchronisation of nematode populations.

A synchronised population of nematode worms ensures that any variability in the susceptibilities of the individual larval stages to the pathogen, is controlled by giving a population that is at the same growth stage. This was achieved by washing a mixed population through a 5 . m mesh sieve (Wilson sieves, Nottingham. UK) with K-medium. The sieve allows all L1 stages to pass through but retains the larger larvae. The L1 stages

were recovered through centrifugation at 2000 x g for 15 minutes at 4 .C. After centrifugation most of the supernatant was carefully removed and the remaining suspension containing the worms used to inoculate plates of NGM pre-seeded with *E. coli* OP50. After 3 days at 16 .C (not including the day of plating) the population will have reached the L4 larval stage, which was then used in the subsequent challenge experiments.

Alternatively nematode populations can be synchronised using a hyper-chlorite treatment that disrupts gravid females to release viable eggs (Sulston and Hodgkin 1988). Briefly, a mixed population of nematodes was washed from a 9 cm NGM plate and centrifuged at 1000 x g for 5 minutes to recover the suspended nematodes. The supernatant was discarded to leave 3.5 ml containing the nematodes. The worms were then disrupted by the addition of 1 ml of a 5% (v/v) hyper-chlorite solution along with 0.5 ml of a 0.5 M NaOH solution. The suspension was then vortexed every 2 minutes for a total of 10 minutes. The suspension was again centrifuged and 4.5 ml of the supernatant discarded. A 4.5 ml aliquot of dH_20 was then added, which helps to further disrupt the gravid females through the change in osmotic potential. The eggs were recovered through centrifugation at 1000 x g for 10 minutes. A 4 ml aliquot of the supernatant was removed leaving 500 . 1 containing the nematode eggs. This was then added to K-medium containing no bacterial food supply and incubated at 16 .C overnight on a rotary platform. Development of the nematodes becomes arrested at the L1 stage. When these arrested L1 larvae are recovered through centrifugation as described above and transferred to a plate pre-seeded with *E. coli* OP50 they continue with their development. After 3 days at 16 .C the population reached the L4 stage

2.2.2. Preparation for and examination of nematode worms by electron microscopy.

2.2.2.1. Examination of *C. elegans* by thin-section transmission electron microscopy.

Nematodes were fixed for 24 hours with 5 % (v/v) glutaraldehyde (Agar Scientific, UK) in 0.1 M phosphate buffer (pH 6) (Taab Laboratories Equipment Ltd., UK.). They were then pelleted by centrifugation at 1000 x g for 10 minutes and the supernatant removed before they were resuspended in 0.1 M phosphate buffer (pH 6). Nematodes were stored in buffer at 4-8 °C for approximately 21 days while sterility checks were undertaken prior to their visualisation by transmission electron microscopy (TEM).

Samples containing nematodes were centrifuged at 1000 x g for 15 minutes and the supernatant removed. Nematodes were then resuspended in 1 % (w/v) osmium tetroxide (Agar Scientific, UK.) in 0.1M phosphate buffer (pH 6) (Taab Laboratories Equipment Ltd., Aldermaston, Berks.) for 4 hours at room temperature, with intermittent agitation. Nematodes were again centrifuged at 1000 x g for 15 minutes and the secondary fixative supernatant removed. The nematode pellet was then gently mixed with molten agar (2 % w/v Oxoid no.1). This was allowed to cool and when solid, cut into approximately 1mm cubes using a razor blade. The agar cubes containing the nematodes were then dehydrated through a graded ethanol series at room temperature according to the following schedule:

30% ethanol – 10 minutes.

50% ethanol – 10 minutes.

70% ethanol – 20 minutes.

90% ethanol – 30 minutes.

100% ethanol – 2 x 30 minute changes.

(all timings approximate)

The agar cubes were then immersed in 100% propylene oxide (Taab Laboratories Equipment Ltd., Aldermaston, Berks.) for 2 x 10 minute changes.

Araldite® resin mixture used for final embedding of samples was prepared as follows: for 10 ml total volume, 5 ml dodecenyl succinic anhydride (DDSA) hardener was added to 5 ml Araldite® CY212 with thorough mixing. 0.2 ml N-benzyldimethylamine (BDMA) accelerator (all resin components from Agar Scientific, UK.) was then added, again with thorough mixing. The final resin mixture was allowed to stand for approximately 1 hour to allow the escape of air bubbles introduced by the mixing process.

Agar cubes were immersed in 50:50 propylene oxide: Araldite® resin mixture at room temperature in sealed tubes for approximately 18 hours. After incubation the cubes were then transferred to 100 % Araldite® resin mixture in open tubes and incubated for approximately 8 hours at room temperature.

Each agar cube was placed in an embedding capsule (BEEM no. 0 - Agar Scientific, UK.) and covered with fresh resin mixture. A unique sample-identifying label was inserted into each capsule. Capsules were then allowed to stand for approximately 60 hours at room

temperature to ensure thorough infiltration of the nematodes by the resin. Embedding capsules were then held at 60 °C . 5 °C for approximately 72 hours to polymerise the resin.

Polymerised resin blocks were removed from the embedding capsules. Using a hand held glass knife, clear resin was trimmed away from the tip of each block to expose the embedded nematodes. The roughly trimmed block face was then smoothed using an ultramicrotome (Reichert-Jung, model Ultracut E – Leica Microsystems UK Ltd. UK.) fitted with a glass knife. The edges of the smoothed block face were then shaped into a truncated pyramid with sides of maximum length 1 mm. Blocks were returned to the ultramicrotome and thin-sections (. 70 nm. thickness) prepared using a diamond knife (Diatome, Switzerland) set at a clearance angle of 5 °. Thin-sections were picked up on 3 mm, 300 mesh copper EM specimen grids held in tweezers.

The grids with adherent thin-sections were immersed in 2 % (w/v) aqueous uranyl acetate solution (Agar Scientific, UK.) for 2 hours followed by three 30 second washes in dH_20 then 30 minutes in 0.1 % (w/v) lead citrate (Ultrastain 2 - Leica Microsystems UK Ltd., UK.). Grids were then washed in a further three times in distilled water, placed on filter paper (No.1 - Whatman International Ltd., Maidstone, Kent) and allowed to air dry. Grids with stained sections were then coated with . 10 nm. of carbon in a vacuum coating unit (model 208 - Cressington Scientific Instruments Ltd., UK.).

Grids were examined in a model CM100 transmission electron microscope (FEI / Philips, Eindhoven, The Netherlands) operated at appropriate magnifications at 80 kV accelerating voltage and using a 15 . m thin-film gold objective aperture. Micrographs were recorded on Ilford EM technical film (Ilford Ltd., UK.) which was processed in Dektol developer (Kodak plc, Chalon-sur-Saone, France). Photographic prints were

prepared from micrographs on Ilford multigrade IV photographic paper using a Durst L1200 enlarger (Durst, Bolzano, Italy) and an Ilford 2150RC processor. These prints were scanned at 100 dpi resolution on a flat-bed scanner (SnapScan 1236 - AGFA UK Ltd., UK.) to produce digital image files which were allocated a unique identifying filename and saved in tagged image file format (tiff).

2.2.2.2. Examination of *C. elegans* by scanning electron microscopy.

Nematodes were initially fixed through the procedure already described for TEM in section: 2.2.2.1. before final processing for visualisation through scanning electron microscopy (SEM).

Poly–l-lysine hydrobromide (Sigma-aldridge.UK) coated 10 mm diameter glass cover slips (Agar Scientific, Bishops Stortford, Herts.) were placed on moist filter paper (No.1 - Whatman International Ltd., UK) in an open glass petri dish. Approximately 0.1ml aliquots of nematode suspension were placed on the cover slips to form a shallow meniscus. The petri dish was then covered and incubated overnight at room temperature to allow nematodes to settle from suspension and attach to the surface of the coverslips. After incubation the cover slips were immersed in 1 % (w/v) osmium tetroxide in 0.1 M phosphate buffer (pH 6) (Taab Laboratories Equipment Ltd., UK.) for 2 hours at room temperature for secondary fixation. The cover slips were then dehydrated through a graded ethanol series at room temperature according to the following schedule:

25% ethanol – 15 minutes.

50% ethanol – 15 minutes.

75% ethanol – 15 minutes.

100% ethanol – 2 x 30 minute changes.

(all timings approximate)

After dehydration the cover slips were immersed in hexamethyldisilazane (HMDS - Taab Laboratories Equipment Ltd., UK.) for 2 x 15 minute changes. Cover slips were then removed form the HMDS using tweezers and gently agitated in air to encourage rapid drying. Dried cover-slips were then mounted on ½ inch aluminium SEM pin stubs (Agar Scientific, UK.) using double-sided adhesive carbon discs (Agar Scientific, UK.). The base of each stub was inscribed with a unique identifying specimen code.

An approximate 10 nm thick coating of gold was applied to the surface of each stub with affixed cover slip using an ion beam vacuum coating system (Model 800 – Atom Tech Ltd., UK). The stubs were rotated and tilted during the coating process to ensure an even and continuous gold layer on the specimens.

Specimen stubs were examined in a model XL30FEG scanning electron microscope (FEI / Philips, Eindhoven, The Netherlands) operated at appropriate magnifications at 4 kV accelerating voltage and using a 30 . m platinum final lens aperture. Digital micrographs were recorded at 800 x 600 resolution, allocated a unique identifying filename and saved in tagged image file format (tiff). Analogue micrographs were recorded on Kodak T-max 100 120 roll film (Kodak plc, Chalon-sur-Saone, France) which was processed in Kodak T-max developer. Photographic prints were produced on Ilford multigrade IV photographic paper

(Ilford Ltd., Mobberley, Cheshire) using a Durst L1200 enlarger (Durst, Bolzano, Italy) and an Ilford 2150RC processor.

2.3. Molecular biological methods.

2.3.1. Isolation of bacterial genomic DNA.

Bacterial genomic DNA was isolated from overnight bacterial cultures of *B. pseudomallei* through the use of a Gentra sytems (Minneapolis, MN, USA) genomic DNA isolation kit following the manufacturers instructions for isolation of DNA from Gram-negative bacteria. Briefly bacterial cells are recovered by centrifugation followed by resuspending in an anionic detergent containing a DNA stabiliser. The suspension was then incubated at 50 .C overnight during which cell lysis occurs. After incubation contaminating RNA was removed by addition of RNAase. Extraneous protein was then removed through salt precipitation and genomic DNA recovered through precipitation in alcohol. DNA was then resuspended in distilled water and stored at –20 .C until required.

2.3.2. Agarose gel electrophoresis.

Agarose was disolved in 1 x Tris Acetate EDTA (40 mM Tris, 20 mM acetic acid, 1 mM EDTA pH 8.0) (TAE) buffer to create a solid gel matrix in which to run DNA samples. Ethidium bromide was also added before pouring of the gel to a final concentration of 0.5 . g/ml. A 1 % (w/v) agarose gel was used for DNA fragments smaller than 1 kb and 0.7 % (w/v) for fragments larger than 1 kb. To aid in loading of gels, to each sample 2 . l of 6 x gel loading buffer was added (Sigma-aldridge, UK). Gels were run at 80-90 volts and visualised using ultraviolet light (310 nm) and a Multigenius Bio-imaging system (Syngene, Cambridge, UK).

2.3.3. Isolation of plasmid and cosmid DNA.

Plasmid DNA was isolated using Qiagen tips according to the manufacturer's instructions for plasmid isolation. Cosmid DNA was also recovered from host bacterial cultures using Qiagen tips, following the manufacturer's instructions for isolation of low copy number plasmids and cosmids (Qiagen, UK). This method of extraction used alkaline lysis to disrupt the bacterial cell membrane. The cell debris was removed and the supernatant passed through silica columns that bind supercoiled DNA. Plasmid and cosmid DNA was then eluted from the column into a low salt buffer (Qiagen miniprep kit) or into high salt buffer (Qiagen maxiprep kit). Purified DNA was then quantified by spectrophotometry at 260 nm and visualised by electrophoresis. Vector DNAs used in this study are detailed in Table: 2.2.

Vector	Genotype	Comments	Source
pUC18	Amp^r	Cloning vector containing MCS with a . - galactocidase gene	Stratagene,
Supercos-1	Amp^r	7.9 kb cosmid vector that accepts 30–42 kb inserts. Dual *cos* sites allow high-efficiency cloning of non-size-selected DNA.	Stratagene

Table: 2.2. Vector DNA used in this study. Physical maps of these vectors showing the restriction endonuclese cutting sites used for cloning in this study can be found in the appendix.

2.3.4. Restriction digestion of DNA.

Restriction endonuclease digestion of DNA was performed using endonuclase restriction enzymes (Roche, UK.) These enzymes cleave at specific nucleotide sequences within the DNA molecule facilitating controlled cleavage and cloning of DNA. A typical digestion contained 2-3 . g of DNA and 20 U of enzyme (10 U/. l). The digests were performed in 20 . l reactions at the temperature required by the endonuclease enzyme and the corresponding buffer solution. Digested DNA was visualised on an agarose gel as described above.

2.3.5. Ligation of DNA fragments.

Ligation of plasmid vector and endonuclease digested DNA fragments was performed using a rapid DNA ligation kit (Roche, UK.) following the manufacture's standard protocol. Briefly, the chosen vector was dephoshorylated using alkaline phosphatase. This prevents recircularisation through removal of phosphate groups from cohesive termini. Samples of 50 ng of vector DNA and 150 ng of insert DNA were mixed in a 10 µl volume. The addition of a ligase enzyme facilitates the ligation of insert into the plasmid vector.

2.3.6. Transformation through heat-shock.

Ligated DNA was transformed into XL10-Gold ultra-competent cells (Stratagene, UK) using heat-shock following the manufacturer's transformation protocol. Briefly, β-mercaptoethanol was added to 100 µl aliquots of competent cells. This increases the transformation efficiency of the reaction. The cells were then incubated on ice for 10 minutes. Following incubation 2 µl of a ligation mixture was added to the mixture and the tubes again incubated on ice for 30 minutes. The cells were then heat pulsed at 42 °C for 30 seconds, which facilitates passage of plasmid DNA into the bacterial cell. Cells were recovered in SOC broth for 1 hour at 37 °C with constant shaking at 200 rpm before plating onto the appropriate selective media.

2.3.7. Transformation through electroporation.

Supercos-1 was electroporated into *E. coli* XL1-Blue MR electro-competent cells (Stratagene) using a Gene Pulser (Bio Rad Laboratories Ltd.). Electroporation cuvettes (0.2 cm gap), SOC medium, Polypropylene pots were chilled on ice. Between 50-500 ng of cosmid DNA was mixed with 40 . l of cells. The cuvette was dried and pulsed in the electroporation chamber at 2.5 kV, 200 Ω using a 25 . F capacitor. One ml of SOC medium was mixed with the cells immediately, placed in a polypropylene tube and incubated at 37 °C, 250 rpm shaking for 1 hour. The cells were then plated onto selective medium and incubated overnight.

2.3.8. Sodium dodecyl sulphate-Polyacrilamide gel electrophoresis (SDS-PAGE).

Analysis of protein expression from cosmids within *E. coli* XL1 Blue MR containing *B. pseudomallei* 576 genomic DNA was achieved by SDS-PAGE. Both the upper stacking and lower separating gel were cast using the method described by Laemmli (Laemmli 1970) using a casting cassette (BioRad. UK). This was then wrapped in laboratory sealing film and stored at 4 °C until needed.

Overnight cultures of the cosmid clones were centrifuged at 6000 x g for 10 minutes. The supernatant was discarded and the resulting pellet resuspended in phosphate buffered saline (PBS) to obtain a viscous solution. A 100 µl aliquot of β-mercaptoethanol and 7 µl of bromophenol blue were added to 2 ml of LPS solubilisation buffer (10 % v/v glycerol, 5 % v/v 2-. mercaptoethanol, 3 % w/v sodium dodecyl sulphate, 62.5 mM tris-HCl pH 6.6, 0.01 % w/v bromophenol blue). Aliquots of 3 µl of the resuspended bacteria were then added to 30 µl aliquots of the mixture and incubated at 100 °C for 5-10 minutes.

The gel was loaded with 8 µl of each sample along with 8 µl of high range rainbow molecular weight marker (Amersham Biosciences, UK) (Appendix: 3.). Electrophoresis was carried out in SDS-PAGE running buffer (Glycine 1.44 % (w/v), SDS 0.1% (w/v), Tris-HCl 0.36 %) at 120 V for approximately 2 hours.

2.3.8.1. Staining with Coomassie Brilliant Blue.

Following SDS-PAGE, proteins were visualised by staining with Coomassie Brilliant Blue (Coomassie Brilliant Blue 5 g/l, methanol 40 % (v/v), glacial acetic acid 10 % (v/v), dH_20 500 ml). Gels were soaked in Coomassie blue solution for 1-2 hours with constant gentle agitation on a rotary platform. Unbound dye was removed by repeated washing with destain solution (methanol 400 ml, glacial acetic acid 100 ml, dH_20 500 ml). The gels were subsequently photographed using an Olympus C-3030 Zoom digital camera on a light box and the resulting images adjusted for colour balance using the computer programme Microsoft photo editor version 3.0.

2.3.8.2. Western blotting.

A section of Immobilon-P (Polyvinylidene Difluoride. PVDF) membrane (Sigma-aldridge, UK) was then pre-cut to the size of the gel and activated by soaking in methanol for 30 seconds and washed in dH_20. Two sheets of blotting paper were also cut to the size of the SDS-PAGE gel and soaked in transfer buffer (150 mM glycine, 25 mM tris-HCl, 10 % v/v methanol, pH 8.3) for 5 minutes. One sheet of pre-soaked blotting paper was laid on the transfer surface of a BioRad-transblot SD-semidry transfer cell (BioRad, UK) and the SDS-PAGE gel placed on top followed by the second piece of blotting paper. The proteins were then transferred to the membrane for 20 minutes at maximum volts and 200 mA.

After transferral of the proteins, the membrane was blocked in 5 % blotto (50 g/l - Skimmed milk powder in PBS) for 5 minutes. The membrane was then washed a further 3 times in 0.1 % blotto for 10 minutes. After washing, the primary antibody diluted 1 in 500 in 0.1 % blotto was added to the membrane and allowed to bind for 1 hour at room temperature. The primary antibody solution was then discarded and the membrane washed again in 0.1 % blotto 3 times for 10 minutes.

The secondary antibody diluted 1 in 2000 in 0.1 % blotto was then added to the washed membrane and allowed to bind at room temperature for 45 minutes. The secondary antibody solution was then also discarded and the membrane washed 3 times for 10 minutes in 0.1 % blotto followed by a further 3 times for 10 minutes in PBS to remove any excess blotto.

Then membrane was then developed by washing in 3, 3'-Diaminobenzine (DAB) peroxidase substrate (Sigma-aldridge fast DAB with metal enhancer, Sigma-Aldridge, UK.) following the manufacturers instructions. Once developed the membrane was washed in distilled water and allowed to air dry overnight.

2.3.8.3. Silver staining.

Electrophoresed gels were fixed for 0.5 hours - overnight in a solution containing 40 % (v/v) methanol, 5 % acetic acid and 55 % deionised water. Gels were oxidised for 5 minutes in the fixing solution with 0.7 % (w/v) periodic acid. Gels were washed with 3 changes of dH_20 each of 15 minutes. Gels were then stained for 10 minutes with silver staining solution (2 ml of ammonium hydroxide [specific gravity 0.88] was added to 28 ml

of 0.1 M sodium hydroxide with stirring and 1 g of silver nitrate dissolved in 5 ml of distilled water was added dropwise while stirring). After staining the gels were then again washed 3 times for 15 minutes in distilled water and developed with 0.05 % (v/v) formalin and 0.005 % (w/v) citric acid in distilled water. The developing solution was then removed and stop solution (5 % acetic acid) was added and allowed to incubate at room temperature for 15 minutes. Prior to visualisation, gels were soaked in distilled water for a minimum of 0.5 hours.

2.4. Assays using nematodes.

2.4.1. The plate-based mortality assay.

Bacterial lawns were first created by placing 10 . 1 of an overnight culture of a pathogen onto the centre of each of three 3.5 cm petri-dishes (Corning, NY) containing NGM media. This was also carried out for *E. coli* OP50 for use as a control. The plates were then incubated at 37 .C overnight. Groups of 10-15 L4 larvae were transferred to the centre of each lawn under microscopy at x 40 magnification, using a . . 1 inoculation loop. The plates were then incubated at 25 °C for a total of 96 hours. The numbers of dead nematodes on the plates containing pathogen and *E. coli* OP50 were recorded daily. This allowed the mortality in each population over time to be calculated. Worms that had inadvertently stuck to the side of the plate and died were discounted from the experiment. Worms were scored as dead if they met the criteria described below

I. No spontaneous movement

II. No response to physical stimuli- knocking the plate

III. Cessation of pharyngeal pumping

IV. Cessation of defecation

V. Appearance as compared to control population

2.4.2. The feeding inhibition assay.

An overnight culture of the bacterial strain of interest was centrifuged at 3000 x g for 15 minutes. The resulting pellet was resuspended in K-medium to an OD_{550} of approximately 1.2. An overnight culture of *E. coli* OP50 was prepared in the same way for use as a feeding control. Two or three 9 cm petri-plates of *C. elegans* were each washed with 5 ml of K-medium and the worm suspension allowed to settle at 4 °C for 15 minutes. The supernatant was removed to leave 2.5 ml of solution containing the nematode worms. The amount of nematodes in three 20 . l aliquots of the worm suspension was then counted, and the mean calculated. This allowed the number of nematodes in the sample to be enumerated and the volume of K-medium in which 50-100 worms were present to be calculated. The suspension was then stored at 4 °C until needed.

The feeding inhibition assay was conducted in 24 well cell culture trays (Costar, Corning. NY). Aliquots of 900 . l of bacterial suspension of the challenge organism was placed into 8 wells, to which was added a volume of gently vortexed worm suspension to give 50-100 nematodes per well. K-medium was then added if necessary to a final volume of 1 ml. A feeding control consisted of 900 . l of *E. coli* OP50 suspension, 50-100 worms in K-medium, again in a final volume of 1ml. A non-feeding control consisted of 850 . l of *E. coli* OP50 suspension, 50-100 worms in 100 . l of K-medium and 50 . l of a 2 M-sodium salicylate solution. Feeding in *P. aeruginosa* PA14 was also often used as a non-feeding control. These wells were set up as described for the test organism or *E. coli* OP50. Three wells without added nematodes were also prepared. These allow the natural degradation of

the bacteria in K-medium to measured and contained K-medium with either the challenge organism, *E. coli* OP50, *E. coli* OP50 and sodium salicylate or *P. aeruginosa* PA14 where necessary. The plates were then incubated at 25 °C on a rocking platform for a total of 48 hours.

At 24 hour intervals, 1 ml samples were transferred from each well into a sterile disposable cuvette (Sigma-aldridge, UK). Cuvettes were incubated at 4 °C for 10 minutes to ensure no worms remained in suspension and the OD_{550nm} of the bacterial suspension measured and recorded. The samples were then returned to their relevant wells through pipetting.

2.5. Investigation of *B. pseudomallei* using *C. elegans*.

2.5.1. Investigation of *B. pseudomallei* interaction with the *C. elegans* gut by electron microscopy

The interaction of *B. pseudomallei* 576 with the *C. elegans* N2 intestine was investigated through the use of TEM (Section: 2.2.2.1.). Overnight cultures of *B. pseudomallei* 576 were centrifuged for 15 minutes at 3000 x g. The pellet was then resuspended in K-medium to a final OD of approx 1.0 at 600nm.This was also performed for *E. coli* OP50 which served as a control.

C. elegans N2 were synchronised to the L4 stage using the method described in section: 2.2.1.4. Ten 9 cm plates of synchronously growing *C. elegans* N2 were washed with 6 ml of K-medium and 5 ml of worm suspension removed. This was completed for the remaining 9 plates to give a total of 50 ml containing the nematodes. The worm suspension

was then centrifuged at 1000 x g at 4 .C for 10 minutes to pellet the suspended nematodes. 45 ml of the supernatant was then carefully removed to leave 5 ml of concentrated worm suspension. This was stored at 4 .C until needed.

To each well of a six well cell culture cluster (Corning , NY) 800 . l of *B. pseudomallei* 576 was added along with 200 . l of nematode suspension. This was also carried out for *E. coli* OP50. The plates were incubated at 25 .C for a total of 24 hours after which the samples were fixed in situ through the addition of 3 ml of 5 % v/v glutaraldehyde. The sample was then transferred to a glass universal bottle and allowed to incubate at room temperature overnight to ensure complete penetration of the fixative.

Samples were washed in phosphate buffer (pH 6) (Taab Laboratories Equipment Ltd., UK) by repeated centrifugation at 1000 x g for 10 minutes. This was repeated a total of four times to remove any remaining glutaraldehyde. The samples were then prepared for visualization by Mr. A.B. Dowsett (Microbial imaging department, Health Protection Agency, Porton down, UK). Examination of the gut lumen may reveal how *B. pseudomallei* interacts with *C. elegans* on a cellular level. Importantly it may also show if the bacterial cells become intracellular during the course of the infection, which may contribute to the pathogenesis in the nematode.

2.5.2. Screening of *B. pseudomallei* transposon mutants using *C. elegans* for a reduction in virulence.

The use of the *C. elegans* nematode model for the investigation of bacterial mutants was explored. A library of transposon mutants of *B. pseudomallei* 576 were investigated using a modified version of the plate-based mortality assay.

Ninety six well cell clusters (Corning, NY) containing 50 µl volumes of LB-broth were inoculated with pools of *B. pseudomallei* 576 transposon mutants using a replica plating tool. These were then allowed to incubate overnight at 37 °C. The resulting cultures were then used to replicate the pools to further 96 well cell clusters containing 50 µl volumes of NGM agar which were again incubated overnight at 37 °C.

Four 9 cm plates containing *C. elegans* N2 previously synchronised to the L4 larval stage were washed with 5 ml of K-medium. The resulting nematode suspension was then allowed to incubate at 4 °C for 20 minutes. After incubation 18 ml of K-medium was removed leaving 2 ml containing the nematodes. Aliquots of 5 µl containing 5-10 worms were then added to each well of the 96 well cell cluster. After incubation at 25 ° C for a total of 96 hours, each well was inspected for live worms using an inverted microscope. The presence of live worms after 96 hours indicated attenuation in the nematode model of infection.

2.5.3. Screening of *B. pseudomallei* cosmid libraries using *C. elegans*.

A *B. pseudomallei* cosmid library was obtained in *E. coli* XL1-Blue MR, which was screened for increased pathogenicity phenotypes using the feeding inhibition assay. Single colonies were obtained by plating 100 . l of 10^{-5}, 10^{-6} and 10^{-7} serial dilutions of an overnight culture of the library onto LB-agar plates containing ampicilin at a concentration of 25 . g/ml. The supercos-1 cosmid vector (Stratagene, UK) contains an ampicillin resistance gene (Appendix: 1.). Thus, the selective pressure induced by the addition of the antibiotic ensures the continued presence of the cosmid within the bacterial cells.

After incubation at 37 .C overnight individual cosmid clones were used to challenge *C. elegans* N2 and the mutant *C. elegans phm*-2 in the form of a simplified feeding inhibition assay. Four plates of *C. elegans* N2 were washed with 6ml of K-medium and 5 ml of liquid removed to give a total volume of 20 ml. This was allowed to settle at 4 .C for 20 minutes and 15 ml of the supernatant discarded to give a final volume of 5ml containing the nematodes.

A selection of 18 cosmid clones was selected and grown at 37 .C overnight. After incubation bacteria were centrifuged at 2000 x g and the supernatant discarded. The resulting pellets were resuspended in K-medium to an approximate OD_{550nm} of 1.2.

The strain XL:cos-1 contains the supercos-1 cosmid vector but without a *B. pseudomallei* 576 genomic insert. Hence, XL:cos-1was prepared in the same way for use as a control.

The experiment was carried out in six well culture clusters (Corning, NY). Into each of 18 wells 1.7 ml of the cosmid clone, 200 . l of nematode suspension, 100 . l of K-medium and 4 . l of ampicillin at a concentration of 25 mg/ml was added. A further 3 wells contained XL: cos-1 along with identical volumes of nematodes, K-medium and ampicillin for use as a feeding control. Finally a non-feeding control was set up in triplicate through the addition of 1.7 ml of XL: cos-1, 200 . l of nematode suspension, 4 . l of ampicillin and 100 . l of a 2M sodium salicylate solution in place of K-medium.

The plates were incubated at 25 .C for a total of 48 hours. At 24 hour intervals, 1 ml samples were transferred from each well into a sterile disposable cuvette (Sigma-aldridge, UK). Cuvettes were incubated at 4 °C for 10 minutes to ensure no worms remained in suspension and the OD_{550nm} of the bacterial suspension measured. After the results had been recorded the samples were returned to their relevant wells for the remainder of the experiment.

Any of those clones seen to inhibit feeding were selected for further study in a standard feeding inhibition assay. Sequencing of fragments from the cosmid insert of interest may allow the identification of those genes or virulence determinants responsible for feeding inhibition and pathogenicity in *C. elegans*.

2.6. Mouse infections and competition assays.

Overnight cultures of the mutant 6H2 and the wildtype strain *B. pseudomallei* 576 grown in LB-broth were centrifuged at 3000 x g for 15 minutes. The supernatant was discarded and the resulting pellets washed in PBS twice. The resulting suspensions were the serially diluted to obtain challenge doses of 10^3 and 10^4 colony forming units (cfu) per 100 . l of bacterial suspension (10^4 and 10^5 cfu/ml). This equated to 10 x and 100 x the mean lethal dose (MLD) for *B. pseudomallei* 576 in BALB/c mice respectively.

To confirm titres of bacteria to be administered before animal infections took place, 100 . l aliquots of each suspension were plated out onto LB-agar plates at 10^{-4}, 10^{-5} and 10^{-6} dilutions and then incubated at 37 .C overnight. The resulting colonies were counted and bacterial titres calculated to obtain the exact numbers of bacteria within the challenge doses.

Two groups of five 6-8 week-old female BALB/c mice were infected by intraperitoneal (IP) injection with 100 . l of bacterial suspension containing the mutant *B. pseudomallei* 6H2 or *B. pseudomallei* 576 at 10 x and 100 x MLD (10^3 and 10^4 cfu/ml respectively). Each group was then monitored for 21 days and the mortality in each group noted.

Any attenuation of the selected mutant was further investigated and quantified through the use of a mouse competition assay. The mutant *B. pseudomallei* 6H2 and *B. pseudomallei* 576 were grown separately in LB-broth at 37 .C overnight. Bacteria were then washed through repeated centrifugation and resuspension in PBS as already described. Aliquots of

0.5 ml of the mutant and wildtype containing approximately 10^4 cfu/ml were then mixed and added to 9 ml of LB-broth (10^8 cfu total bacteria). Serial dilutions of this suspension were then plated out onto LB-agar at 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions to measure total cfu/ml. Additionally as the mutant carried a kanamycin selectable marker aliquots were also plated out onto LB-agar containing 700 . g/ml of kanamycin to determine the mutant cfu/ml. From this the exact input ratio of mutant to wildtype was calculated.

Aliquots of 100 . l of the mixed suspension containing approximately 10^4 cfu (10^2 cfu/ml of mutant and 10^2 cfu/ml of wildtype) were then used to infect 5 female 6-8 week-old BALB/c mice by IP injection. After 48 hours spleens were recovered through the method described by Pepe and Miller (Pepe 1993). Homogenates were diluted and plated out onto selective and non-selective media to determine the output ratio of the mutant to the wildtype. The competitive index is defined as the output ratio (mutant/wildtype) divided by the input ratio (mutant/wildtype). Regression analysis was then used to determine whether the wildtype/mutant ratio recovered from infected spleens was significantly different from the wildtype/mutant ratio present in the original challenge inoculum.

To exclude the possibility of general growth defects under optimal laboratory conditions an *in vitro* competition assay was also performed. A volume of 4 ml of LB-broth was inoculated with 0.5 ml aliquots of bacterial suspension containing 1 x 10^4 cfu/ml of the mutant 6H2 and 1 x 10^4 cfu/ml of the wildtype (1 x 10^4 total cfu). The input ratio was then determined again through selective plating on LB media and LB media supplemented with kanamycin as already described. The mixture was then incubated at 37 .C overnight with constant shaking at 200 rpm. After incubation the output ratio was determined by selective plating and the competitive index calculated as detailed above.

2.7. Statistical analyses.

Students t-tests to assess significant differences between data were performed on the statistical package MS Exel 97 (P = 0.05). Probit analysis to obtain the time at which 50 % of animals die (TD_{50}), regression analyses and analysis of variance (ANOVA) were performed using the statistical package Mini Tab version 13.0 with an assumed log distribution where appropriate unless otherwise stated.

Chapter 3: Establishment of a nematode model of infection.

3.1. Introduction.

The study of bacterial disease in a suitable animal model is a necessary requirement for the study of bacterial pathogenesis. However the use of higher order animal models is limited by moral and ethical concerns, the logistics and cost of animal husbandry and safety concerns associated with the infection of animals with pathogens such as *B. pseudomallei*. The use of cell culture models may provide a suitable alternative to whole animal model systems. However, the vast majority of these can only provide information on particular aspects of bacterial pathogenesis such as attachment or intracellular survival. Furthermore these systems are not truly representative of the whole animal. They consist of discreet populations of individual cell types and not co-ordinated networks of different tissues working synergistically as would be found in a whole animal (Wallis 1998). Moreover eukaryotic cells are not amenable to facile genetic analysis making the dissection of host-pathogen interactions difficult. An animal model that is cost effective, easy to use and readily genetically manipulated would be preferable, these requirements are met by the nematode *C. elegans*. Thus the nematode has become a widely used alternative model host systems for the study of developmental biology, ageing and bacterial pathogenesis.

Current assay methodologies rely on the observation of mortality of a population of nematodes exposed to lawns of pathogenic bacteria on a solid matrix. However this may not detect small changes that result from an intermediate level of pathogenicity. Jones and Candido (Jones and Candido 1999) first reported a response in *C. elegans* where feeding on a bacterial suspension was inhibited by a variety of chemical stressors. These included alcohols, heavy metals, sulfhydryl-reactive agents such as the fungicide captan and salicylic acid (Jones and Candido 1999). This response provides the nematode with a rapid

means of reducing the intake of detrimental chemicals or toxins thus minimising any damage that may be incurred. The reduction in feeding rate was due to the cessation of pharyngeal pumping used by the nematode to ingest its bacterial food supply. Hence the reduction in feeding rate could be measured by simply monitoring the change in optical density (OD) of the bacterial suspension in which the nematodes feed over time.

I reasoned that the feeding inhibition observed might also occur if nematodes were allowed to feed on pathogenic bacteria. This would then allow the model to be exploited as an assay that would not only rely on the observation of mortality in a small population of nematodes but the response of a large population to a pathogen as a whole. Thus the greater sample size would give an increase in sensitivity and give a quantitative result not open to interpretation by the experimenter, as would be the plate-based mortality assay already described.

The aim of the work reported in this thesis was to investigate the usefulness of the nematode *C. elegans* as an alternative model of bacterial infection for the dangerous human pathogen *B. pseudomallei*. However to allow the establishment of a model of nematode killing by bacterial pathogens, the killing by *P. aeruginosa* PA14 in the standard plate-based mortality assay was initially investigated.

3.2. Killing of *C. elegans* by *P. aeruginosa* PA14.

The killing of *C. elegans* on solid media reported by Tan *et al* (Tan 2002) was first investigated. Wildtype *C. elegans* N2 (var Bristol) were allowed to feed on lawns of *P. aeruginosa* PA14, which had been grown over-night on NGM media. The mortality in the original cohort of 10-15 L4 stage worms over time was noted and the percentage mortality in the population at each time point calculated. This was then compared to the percentage mortality when nematodes fed on *E. coli* OP50.

When fed on *P. aeruginosa* PA14 the first nematode death had occurred at 48 hours post infection. By 96 hours all nematodes had died. This was in contrast to that seen on *E. coli* OP50 which only caused a small increase in percentage mortality over the course of the experiment (Figure: 3.1.).

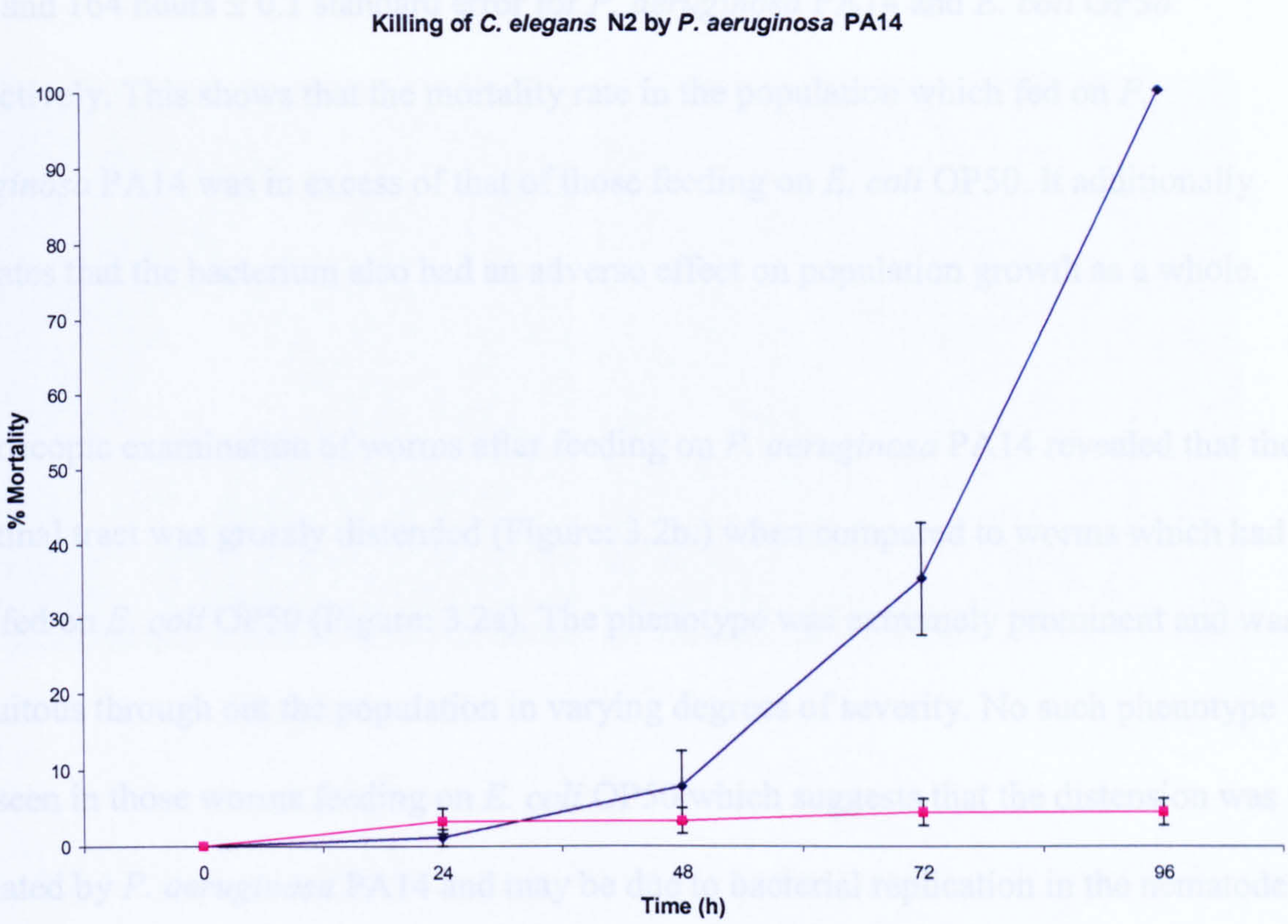


Figure: 3.1. Death of *C. elegans* N2 after exposure to *P. aeruginosa* PA14 or *E. coli* OP50 for 96 hours at 25 °C. Each experiment contained a total number of 9 replicates for each organism, containing 10-15 L4 stage worms. Error bars are the standard error of the mean % mortality. ■ -*E. coli* OP50, ◆ -*P. aeruginosa* PA14.

The difference in killing rate observed was highly statistically significant as shown by a paired students t-test assuming equal variance (P value <0.001). The TD_{50} value while feeding on each bacterium was also calculated. TD_{50} values were 73 hours ± 1.4 standard error and 164 hours ± 0.1 standard error for *P. aeruginosa* PA14 and *E. coli* OP50 respectively. This shows that the mortality rate in the population which fed on *P. aeruginosa* PA14 was in excess of that of those feeding on *E. coli* OP50. It additionally indicates that the bacterium also had an adverse effect on population growth as a whole.

Microscopic examination of worms after feeding on *P. aeruginosa* PA14 revealed that the intestinal tract was grossly distended (Figure: 3.2b.) when compared to worms which had only fed on *E. coli* OP50 (Figure: 3.2a). The phenotype was extremely prominent and was ubiquitous through out the population in varying degrees of severity. No such phenotype was seen in those worms feeding on *E. coli* OP50 which suggests that the distension was mediated by *P. aeruginosa* PA14 and may be due to bacterial replication in the nematode intestine.

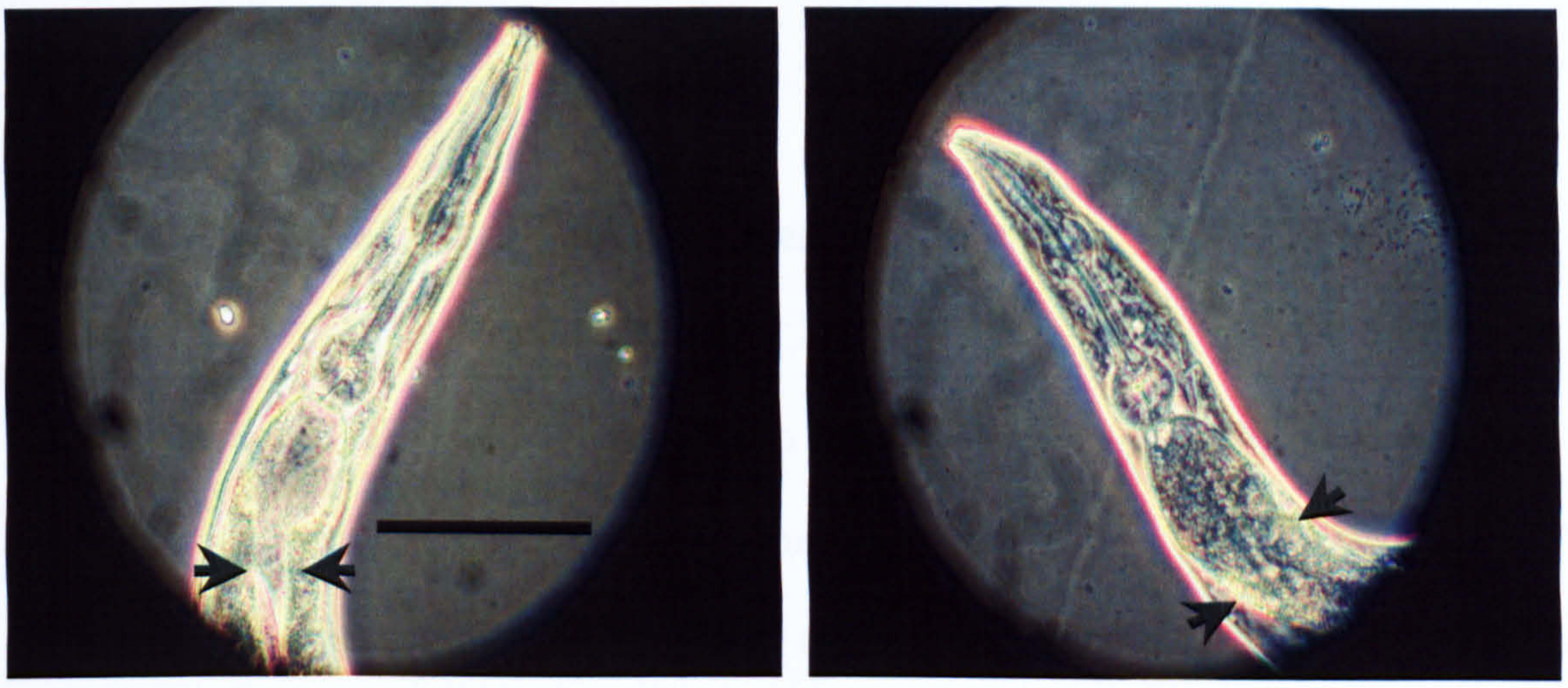


Figure: 3.2. Comparison of the *C. elegans* N2 intestine after feeding on *E. coli* OP50 (a) or *P. aeruginosa* PA14 (b) for 72 hours at 25°C. Arrows delineate the diameter of the intestinal tract. Scale bar represents 50 μm.

The eggs of nematodes exposed to *P. aeruginosa* PA14, were seen to hatch internally before deposition causing death of the worm. This so-called "bag of worms" phenotype was observed in a large majority of those worms killed. This suggests that the phenomenon was bacterially mediated and a consequence of bacterial infection.

3.3. Feeding of *C. elegans* N2 in liquid media

The response of *C. elegans* N2 when fed on lawns of *P. aeruginosa* PA14 showed that the nematode could be killed by the organism on solid media as previously reported (Tan 2002). This represents the standard method for assessment of bacterial virulence using *C. elegans* that will be used in this study. To allow the investigation of novel methods for the measurement of virulence based on the observations of Jones and Candido (Jones and Candido 1999) the response of *C. elegans* when fed *P. aeruginosa* PA14 in suspension was also assessed.

Initially, to allow the level of normal nematode feeding in liquid media to be studied, the feeding of *C. elegans* N2 on bacterial suspensions of *E. coli* OP50 was investigated. The inhibitory effect of 100 mM sodium salicylate solution on bacterial consumption was also assessed. Groups of 100-200 worms were allowed to feed for 48 hours at 25 °C in 2 ml of a bacterial suspension containing either *E. coli* OP50 or *E. coli* OP50 and 100 mM sodium salicylate in K-medium (section: 2.4.2). At 24 hour intervals samples were taken and the OD at 550 nm (OD_{550nm}) of the suspension recorded.

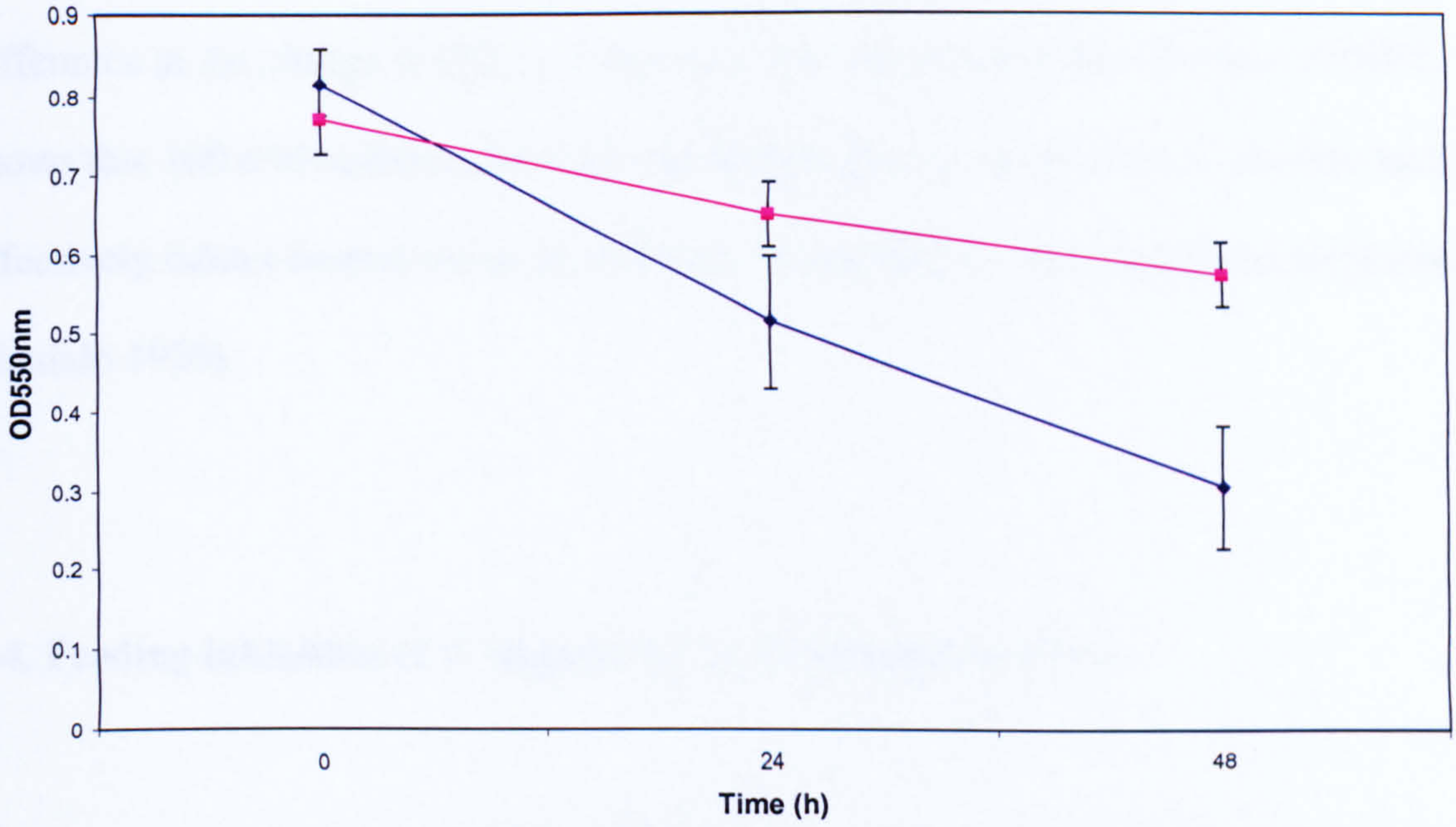


Figure: 3.3. Decline in OD_{550nm} of 2 ml suspensions of *E. coli* OP50 in K-medium at 25 °C due to the feeding of *C. elegans* N2. ◆ - feeding on *E. coli* OP50 alone, ■ - feeding on *E. coli* OP50 and 100 mM sodium salicylate. 10 replicates were performed for each condition containing between 100-200 worms. Error bars are the standard error of the mean OD_{550nm} of the 10 replicates performed.

The OD_{550nm} of each bacterial suspension declined over time due to the feeding action of the nematodes (Figure: 3.3.). This indicates that *C. elegans* can feed in suspension and cause a concomitant drop in OD of the bacterial suspension. The OD_{550nm} of the suspension containing only *E. coli* fell by 0.51 OD units whereas the OD_{550nm} of the suspension containing *E. co*li OP50 and sodium salicylate only declined by 0.19 OD units. The difference in the change in OD_{550nm} over time was significant with a P value of 0.006. This shows that 100 mM sodium salicylate was sufficient to cause stress in *C. elegans* and effectively inhibit normal nematode feeding, as reported by Jones and Candido (Jones and Candido 1999).

3.4. Feeding inhibition of *C. elegans* N2 by *P. aeruginosa* PA14.

As *P. aeruginosa* PA14 caused significant mortality in the plate-based mortality assay the change in OD_{550nm} when worms were incubated with *P. aeruginosa* PA14 at 25 °C was investigated. Groups of 100-200 nematodes were allowed to feed in suspensions of either *E. coli* OP50 or *P. aeruginosa* PA14 in K-medium for 48 hours and the OD_{550nm} recorded at 24 hour intervals (Figure: 3.4.).

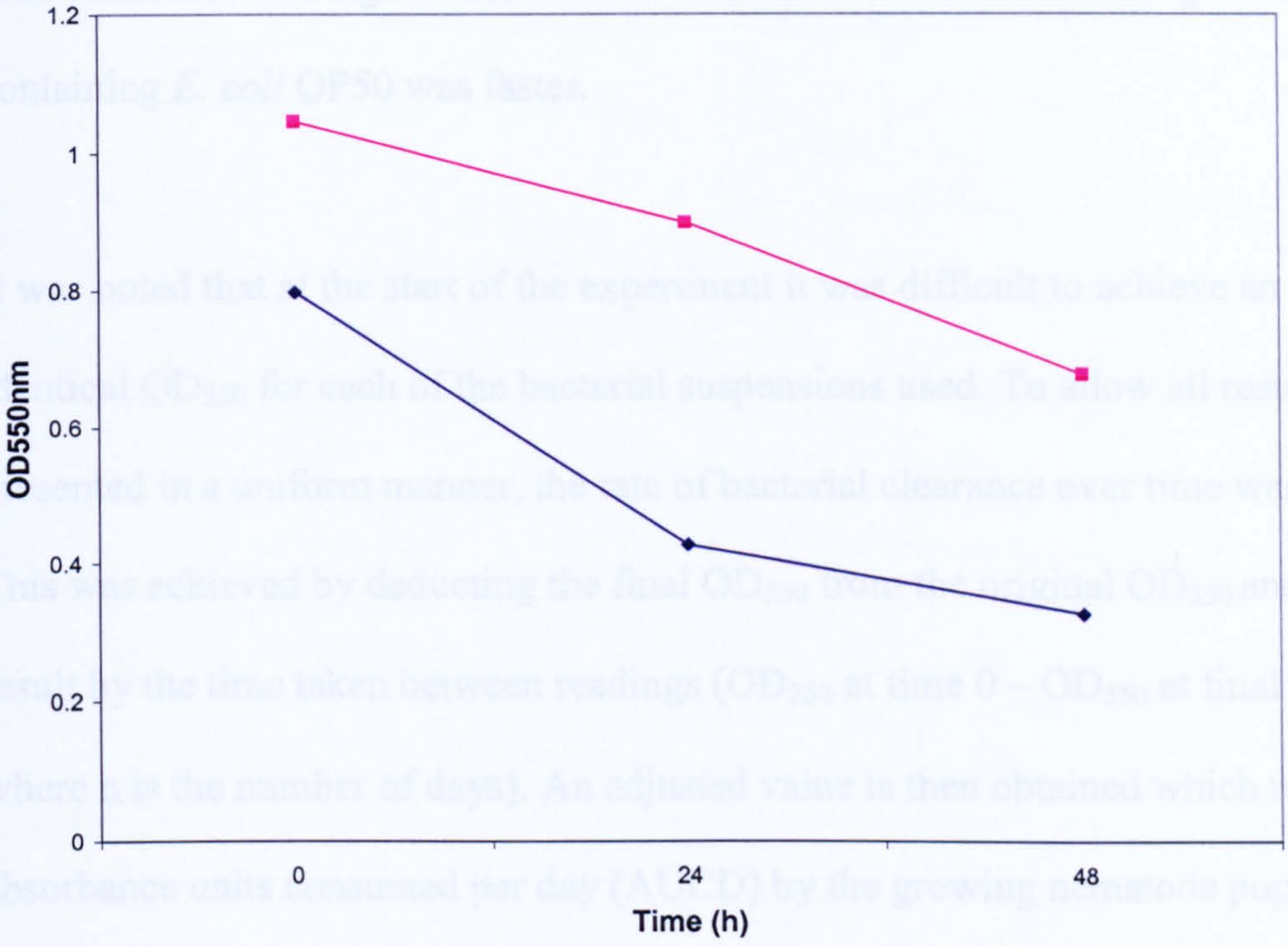


Figure: 3.4. Decline in OD_{550nm} of bacterial suspensions containing *E. coli* OP50 (■) or *P. aeruginosa* PA14 (◆) due to feeding by *C. elegans* N2 at 25 °C. 40 replicates were performed for each strain, containing between 100-200 worms. Error bars are the standard error of the 40 replicates performed. However the standard error only ranges from 0.02 - 0.09 for each strain and hence the error bars are not visible.

Over the course of the experiment the OD_{550nm} of both bacterial suspensions decreased (Figure: 3.4.). By 48 hours the rate of decline of OD_{550nm} between organisms was different. At 48 hours the OD550nm of *P. aeruginosa* PA14 suspensions had declined by 0.37 OD units and the OD550nm of suspensions of *E. coli* OP50 had declined by 0.47 OD units. This difference was significant with a P value of 0.003. Hence feeding in suspensions containing *E. coli* OP50 was faster.

It was noted that at the start of the experiment it was difficult to achieve an exactly identical OD_{550} for each of the bacterial suspensions used. To allow all results to be presented in a uniform manner, the rate of bacterial clearance over time was calculated. This was achieved by deducting the final OD_{550} from the original OD_{550} and dividing the result by the time taken between readings (OD_{550} at time 0 – OD_{550} at final time point/ n, where n is the number of days). An adjusted value is then obtained which represents the absorbance units consumed per day (AUCD) by the growing nematode population feeding in suspension.

It was also reasoned that due to the different characteristics of the bacterial strains used the lysis of the bacteria in K-medium would be different. This would effectively cloud any real reduction in feeding attributable to the pathogen as differences in background cell lysis in suspension would give a false picture of the rate of consumption of bacterial cells. To assess the possibility of different rates of bacterial lysis overnight cultures *of P. aeruginosa* PA14 and *E. coli* OP50 resuspended in K-medium were incubated at 25 °C for a total of 48 hours and the OD_{550nm} of each suspension recorded at 24 hour intervals.

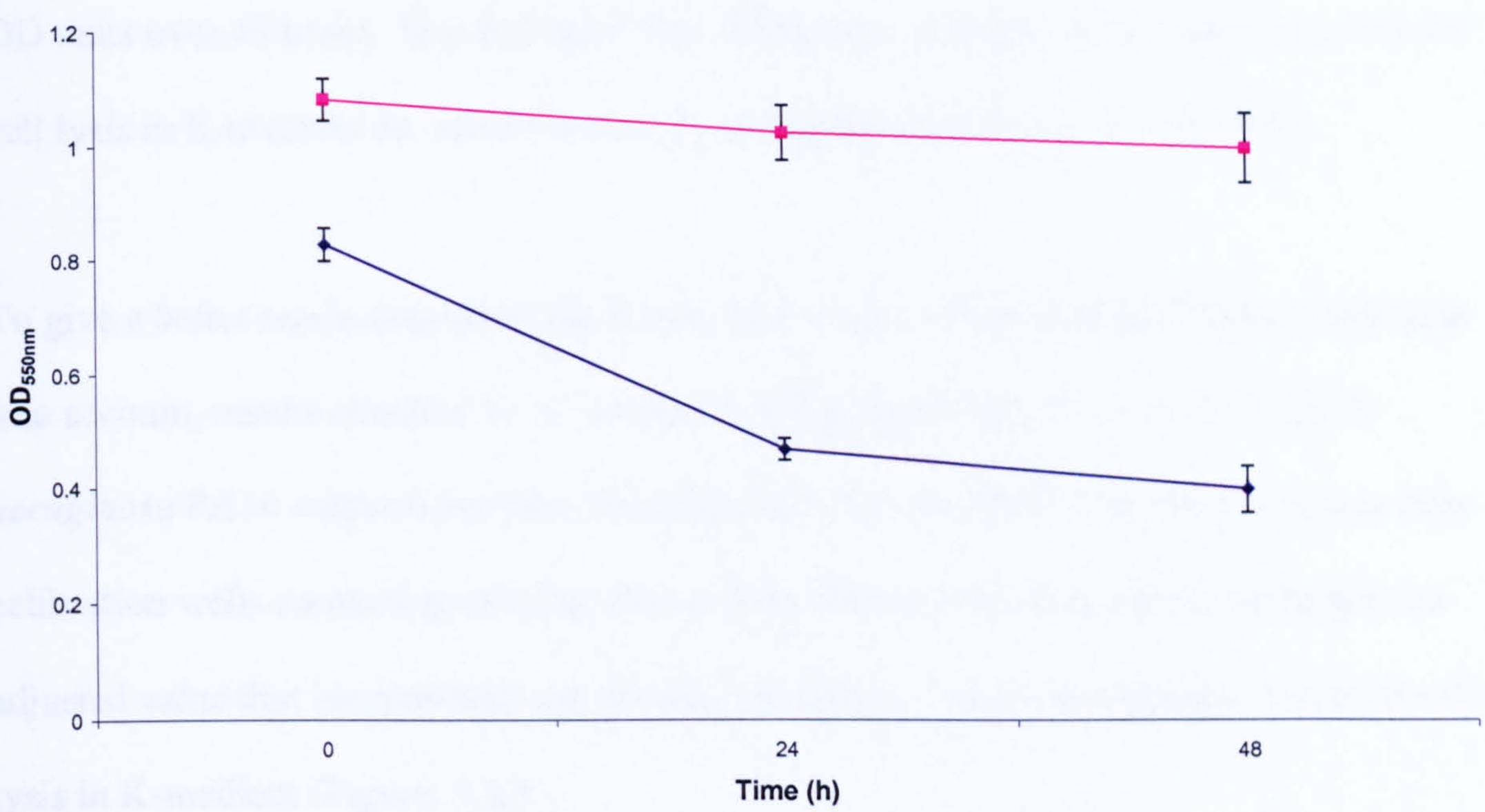


Figure: 3.5. Reduction in OD_{550nm} of suspensions of *E. coli* OP50 (■) or *P. aeruginosa* PA14 (◆) in K- medium at 25 °C over 48 hours. Error bars are the standard error of the mean OD for the 5 replicates performed for each organism.

The rate of reduction in OD_{550nm} of *P. aeruginosa* PA14 in K-medium at 25 °C was faster than *E. coli* OP50 (Figure: 3.5.). The difference in reduction of OD_{550nm} was significant with a P value of 0.0001 at 48 hours. The OD_{550nm} of suspensions containing *P. aeruginosa* PA14 fell over the course of the experiment giving a total decrease of 0.43 OD units at 48 hours. However *E. coli* OP50 was relatively stable in suspension and only declined by 0.08 OD units over 48 hours. This indicated that differences in degradation rates due to natural cell lysis in K-medium do occur between *P. aeruginosa* PA14 and *E. coli* OP50.

To give a better representation of the results and to take any natural background cell lysis into account, results obtained for *C. elegans* N2 feeding in both *E. coli* OP50 and *P. aeruginosa* PA14 suspensions were recalculated to give AUCD. The relevant values from calibration wells containing only bacteria and no worms were then subtracted to give an adjusted value that incorporated any decrease in OD_{550nm} due to background bacterial cell lysis in K-medium (Figure: 3.6.).

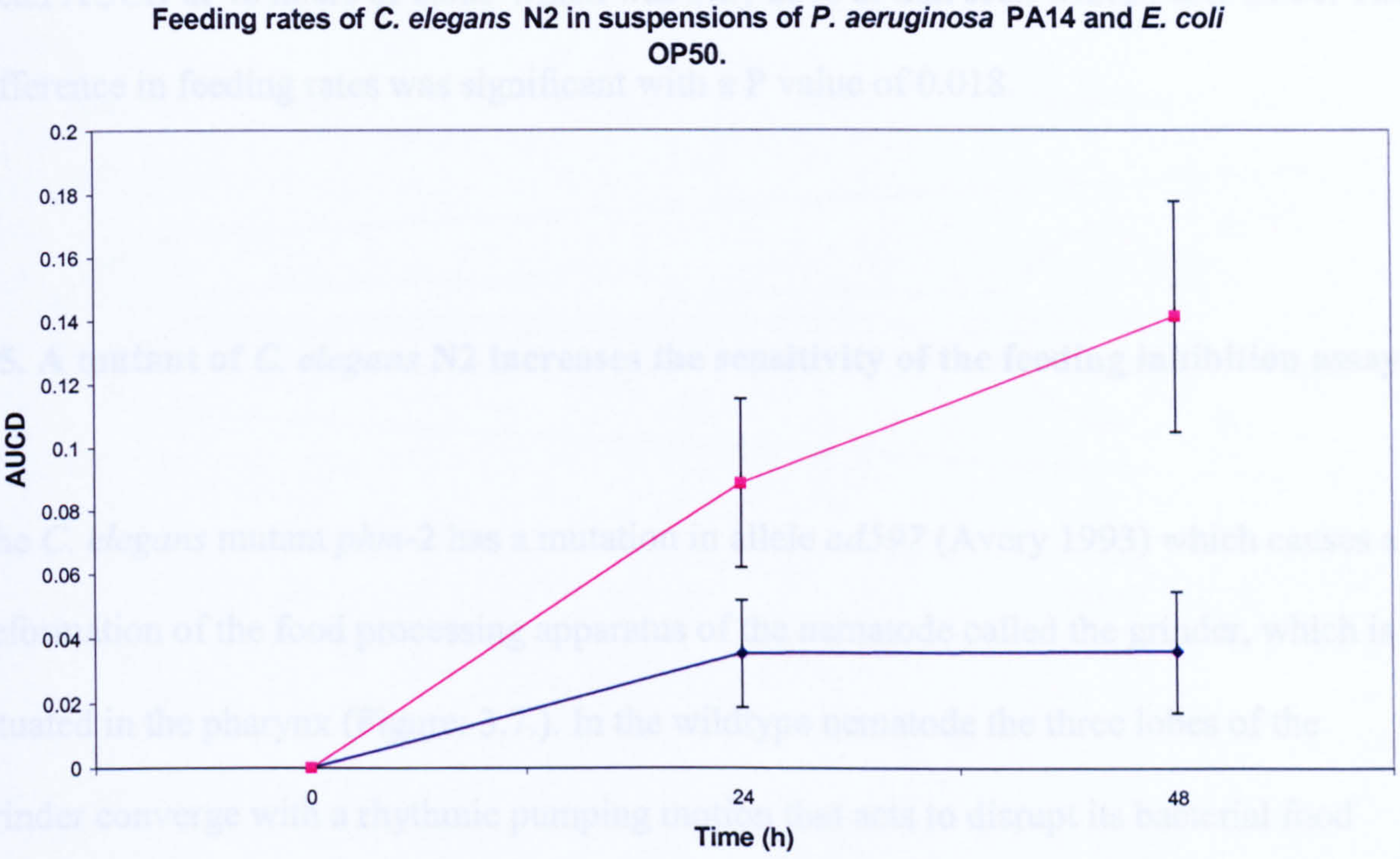


Figure: 3.6. Consumption rates of *C. elegans* N2 on suspensions of *E. coli* OP50 (■) or *P. aeruginosa* PA14 (◆) at 25 °C over 48 hours. 40 replicates were performed each containing 100-200 worms. Results obtained in OD units have been recalculated to give AUCD and further adjusted to allow calibration for background cell lysis in K-medium. Error bars are the standard error of the fully adjusted values of the 40 replicates performed.

The consumption rate on *E. coli* OP50 was seen to increase steadily over 48 hours. Feeding rates were maximal at 48 hours with a mean AUCD of 0.141. However consumption of *P. aeruginosa* PA14 increased until 24 hours where feeding rate decreased (Figure: 3.6.). Feeding on *P. aeruginosa* PA14 was markedly slower than seen with *E. coli* OP50 with a mean AUCD at 48 hours of 0.035 which was only 25% of that seen with *E. coli* OP50. The difference in feeding rates was significant with a P value of 0.018.

3.5. A mutant of *C. elegans* N2 increases the sensitivity of the feeding inhibition assay.

The *C. elegans* mutant *phm*-2 has a mutation in allele *ad597* (Avery 1993) which causes a deformation of the food processing apparatus of the nematode called the grinder, which is situated in the pharynx (Figure: 3.7.). In the wildtype nematode the three lobes of the grinder converge with a rhythmic pumping motion that acts to disrupt its bacterial food supply before releasing it into the intestine for digestion. However in *C. elegans phm*-2 the grinder cannot come to its full forward position (Avery 1993). This aberrant functioning allows a larger number of viable bacterial cells to enter the intestine. Hence the mutant *phm*-2 is concomitantly more sensitive to bacterial infection than the wildtype nematode, as colonisation of the intestine is not hampered by the mechanical disruption of the grinder.

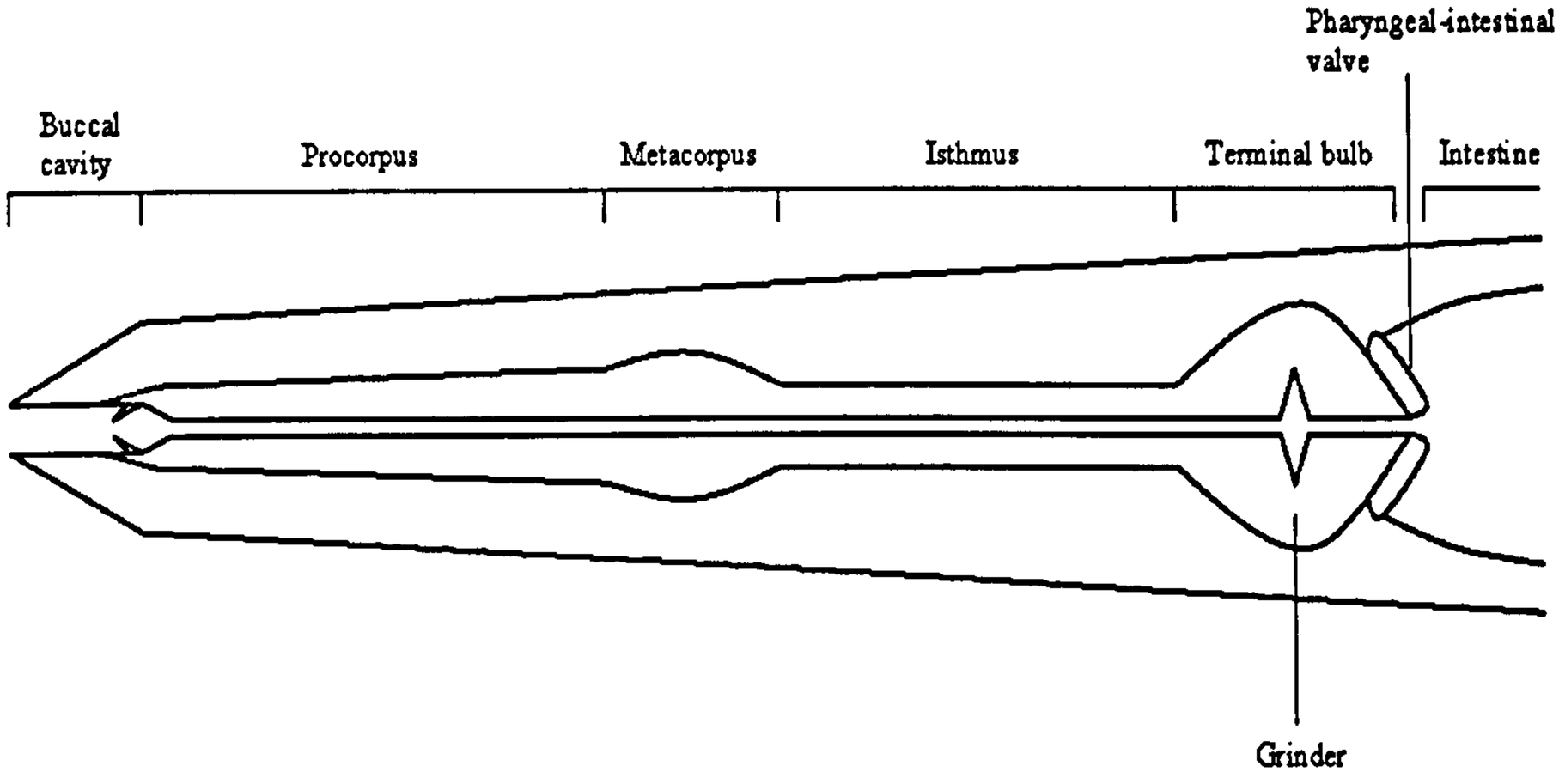


Figure: 3.7. A diagrammatic representation of the pharynx of *C. elegans*. Bacterial cells are passed from the buccal cavity through the metacorpus using rhythmic pumping motion of the attending pharyngeal muscles. Cells are finally disrupted by the grinder within the terminal bulb before being released by the pharyngeal-intestinal valve into the intestine for digestion. Adapted from Wood *et al* 1988 (White 1988).

The mutant *phm*-2 has previously been shown to be more sensitive to killing by *S.enterica* servoar Typhimurium (Labrousse, Chauvet *et al.* 2000). The possibility of this mutation also causing an increase in susceptibility of *C. elegans phm*-2 to infection by *P. aeruginosa* PA14 was investigated through comparison to *E. coli* OP50 in both the plate-based mortality assay and the feeding inhibition assay.

Groups of 10-20 L4 larval stage *C. elegans* N2 and *C. elegans* phm-2 worms were placed on lawns of *P. aeruginosa* PA14 and the mortality at 25 °C in each population monitored over 96 hours. Death of *C. elegans* N2 began at 48 hours and continued until 96 hours when no nematodes remained alive. Similarly the percentage mortality of *C. elegans phm*-2 also increased over the course of the experiment and all nematodes were dead by 96 hours (Figure: 3.8.). However mortality of *C. elegans phm*-2 increased sharply at 24 hours whereas mortality of *C. elegans* N2 only increased markedly at 48 hours. Percentage mortality at 48 hours between the strains was significantly different with a P value of 0.024. This suggests that the mutant *C. elegans phm*-2 is killed at a faster rate than that of the wildtype strain *C. elegans* N2.

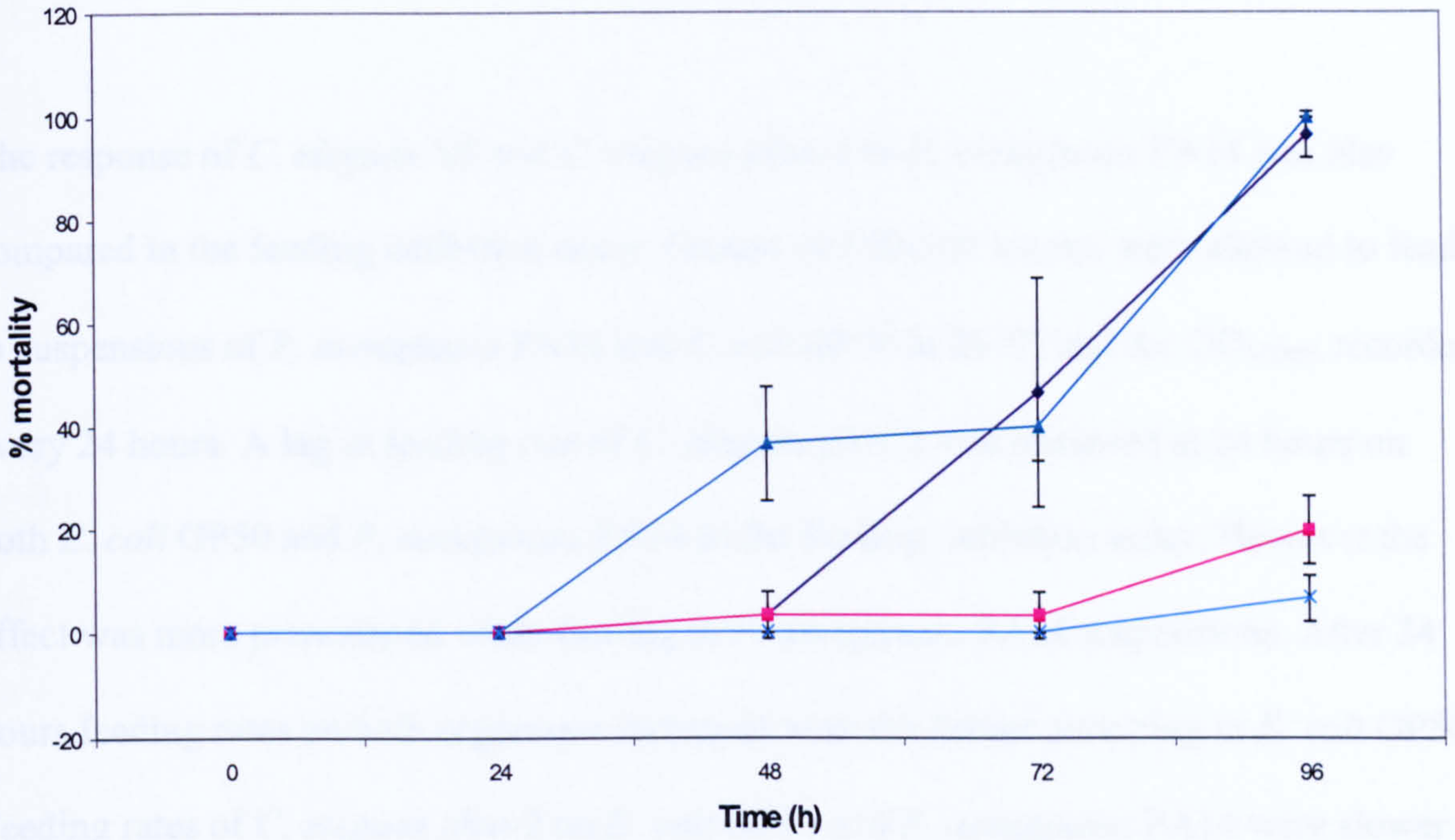


Figure: 3.8. Percentage mortality of *C. elegans* N2 and C. elegans *phm*-2 in the plate-based mortality assay when feeding on *P. aeruginosa* PA14. Feeding on *E. coli* OP50 was used as a control. ◆ - *C. elegans* N2 feeding on *P. aeruginosa* PA14, ■ - *C. elegans* N2 feeding on *E. coli* OP50, ▲ - *C. elegans phm*-2 feeding on *P. aeruginosa* PA14 and × - *C. elegans phm*-2 feeding on *E. coli* OP50. Error bars are the standard error of the mean % mortality from 3 replicates performed each containing 10-20 worms.

TD_{50} values for *C. elegans* N2 and *C. elegans phm*-2 were 73 hours ± 2.9 standard error and 65 hours ± 2.9 standard error respectively. This indicates that the percentage mortality of the wildtype *C. elegans* N2 and the mutant *C. elegans phm*-2 is different when feeding on lawns of *P. aeruginosa* PA14 and that *C. elegans phm*-2 may be more sensitive to killing by this organism.

The response of *C. elegans* N2 and *C. elegans phm*-2 to *P. aeruginosa* PA14 was also compared in the feeding inhibition assay. Groups of 100-200 worms were allowed to feed in suspensions of *P. aeruginosa* PA14 and *E. coli* OP50 at 25 °C and the OD_{550nm} recorded every 24 hours. A lag in feeding rate of *C. elegans phm*-2 was observed at 24 hours on both *E. coli* OP50 and *P. aeruginosa* PA14 in the feeding inhibition assay. However the effect was more pronounced when feeding in *P. aeruginosa* PA14 suspensions. After 24 hours feeding rates on both organisms increased with the fastest occurring in *E. coli* OP50. Feeding rates of *C. elegans phm*-2 on *E. coli* OP50 and *P. aeruginosa* PA14 were slower than *C. elegans* N2 over all. Feeding on *E. coli* OP50 was 64 % slower than that observed for *C. elegans* N2 and 29 % slower compared to wildtype feeding rates on *P. aeruginosa* PA14 (Figure: 3.9.).

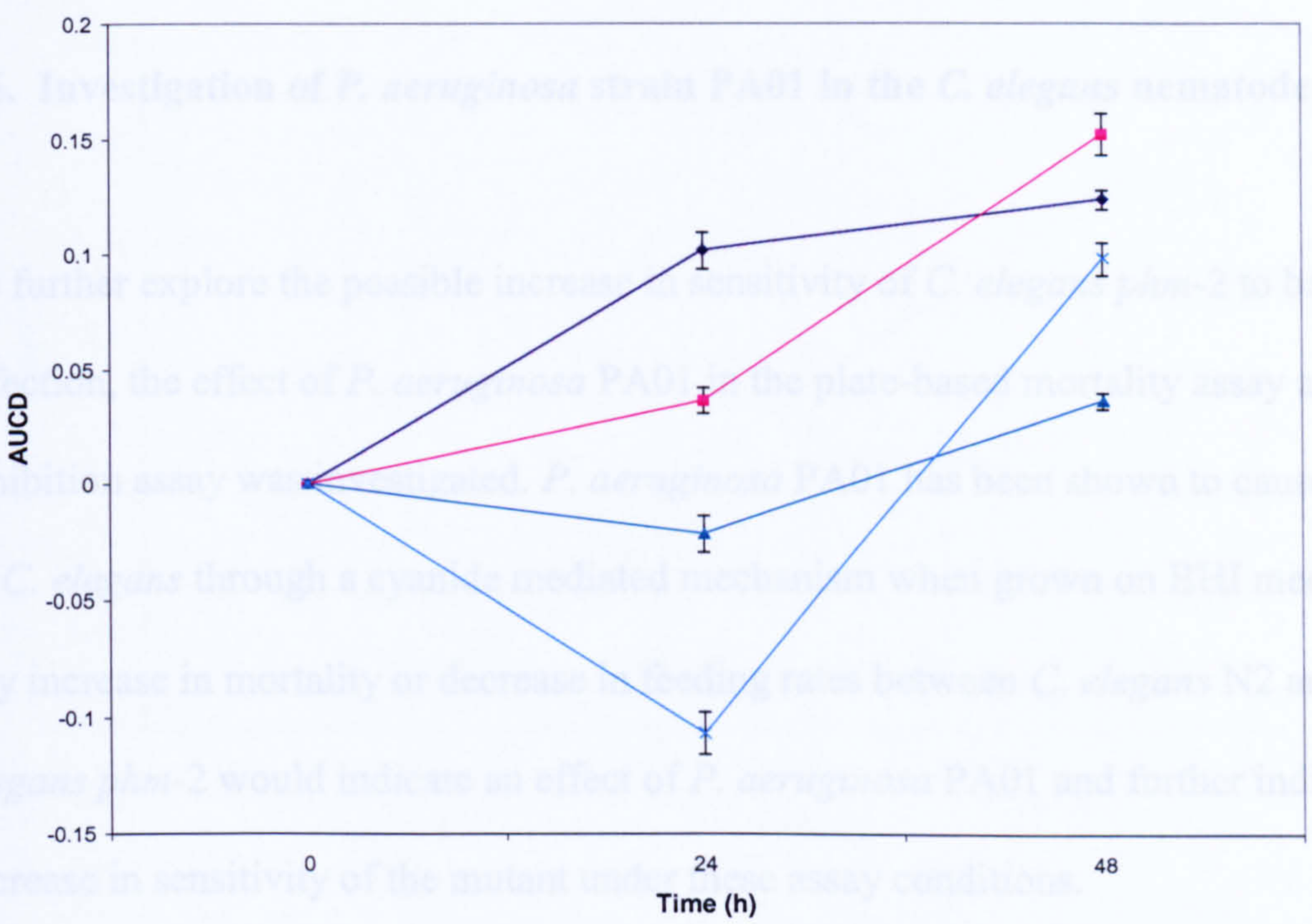


Figure: 3.9. Feeding rates of *C. elegans* N2 and *C. elegans phm*-2 on *P. aeruginosa* PA14 in the feeding inhibition assay at 25 °C. Feeding on *E. coli* OP50 was used as a control. ◆ - *C. elegans* N2 feeding on *P. aeruginosa* PA14, ■ - *C. elegans* N2 feeding on *E. coli* OP50, ▲ - *C. elegans phm*-2 feeding on *P. aeruginosa* PA14 and × - *C. elegans phm*-2 feeding on *E. coli* OP50. Error bars represent the standard error of the mean AUCD from 6 replicates each containing 100-200 worms.

Consumption rates in *P. aeruginosa* PA14 at 48 hours were 0.12 AUCD and 0.03 AUCD for wild type and mutant nematode strain respectively indicating a difference in feeding rates between the nematode strains used. The difference in feeding rates observed in the feeding inhibition assay was significantly different with a P value of <0.001.

3.6. Investigation of *P. aeruginosa* strain PA01 in the *C. elegans* nematode model.

To further explore the possible increase in sensitivity of *C. elegans phm*-2 to bacterial infection, the effect of *P. aeruginosa* PA01 in the plate-based mortality assay and feeding inhibition assay was investigated. *P. aeruginosa* PA01 has been shown to cause the death of *C. elegans* through a cyanide mediated mechanism when grown on BHI media. Hence any increase in mortality or decrease in feeding rates between *C. elegans* N2 and *C. elegans phm*-2 would indicate an effect of *P. aeruginosa* PA01 and further indicate any increase in sensitivity of the mutant under these assay conditions.

Groups of 50-100 worms were allowed to feed on suspensions of either *P. aeruginosa* PA01, *P. aeruginosa* PA14 or *E. coli* OP50 in K- medium at 25 °C for 48 hours and the change in OD_{550nm} was recorded daily. Feeding of the wildtype and mutant worms on *P. aeruginosa* PA01 was slower when compared to feeding on *E. coli* OP50. Feeding rates of *C. elegans phm*-2 on all bacterial strains tested was again slower than that of *C. elegans* N2 (Figures: 3.10. and 3.11.). At 48 hours, consumption rates of *C. elegans* N2 on *P. aeruginosa* PA01was only 66.6% of that seen on *E. coli* OP50. Furthermore feeding of *C. elegans phm*-2 on *P. aeruginosa* PA01 was only 52 % of that observed for *E. coli* OP50. However the difference between consumption rates of *C. elegans phm*-2 on *E. coli* OP50 and *P. aeruginosa* PA01 was not significant (P value = 0.09).

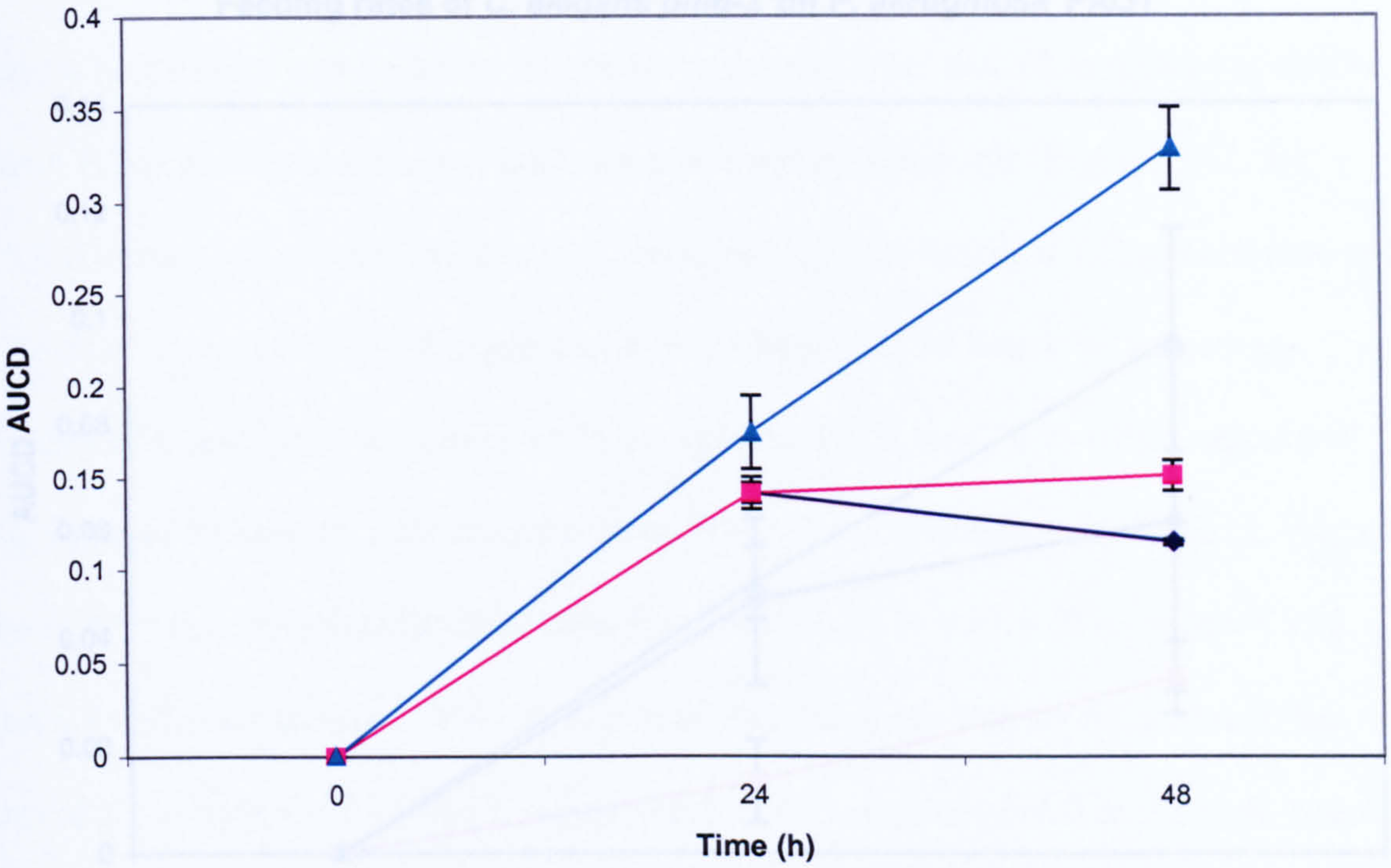


Figure: 3.10. Consumption rates of *C. elegans* N2 when feeding on *P. aeruginosa* PA01 (◆), *P. aeruginosa* PA14 (■) or *E. coli* OP50 (▲) over 48 hours at 25 °C in the feeding inhibition assay. Feeding on *P. aeruginosa* PA14 and *E. coli* OP50 were used as controls. Error bars are the standard error of the mean feeding rate (AUCD) of 6 replicates each containing 50-100 worms.

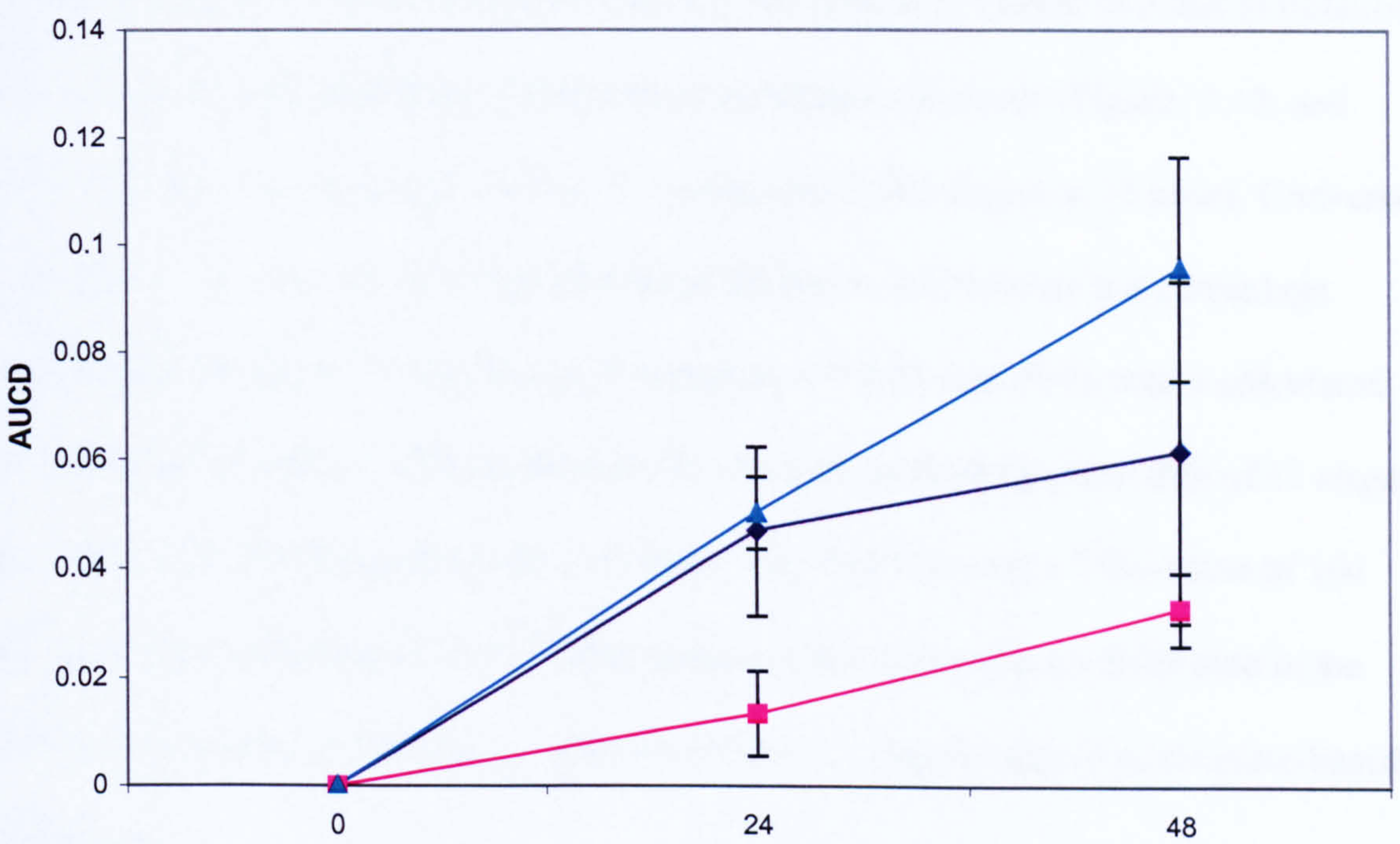


Figure: 3.11. Consumption rates of *C. elegans phm*-2 when feeding on *P. aeruginosa* PA01 (◆), *P. aeruginosa* PA14 (■) or *E. coli* OP50 (▲) over 48 hours at 25 °C in the feeding inhibition assay. Feeding on *P. aeruginosa* PA14 and *E. coli* OP50 were used as controls. Error bars are the standard error of the mean feeding rate (AUCD) of 6 replicates each containing 50-100 worms.

The effect *P. aeruginosa* PA01 on *C. elegans* N2 and *C. elegans phm*-2 was also assessed in the plate-based mortality assay. Groups of 10-20 L4 stage wildtype and mutant nematodes were allowed to feed on lawns of *P. aeruginosa* PA01 for 96 hours at 25 °C and the mortality in each population recorded at 24 hour intervals.

After 96 hours there was no difference between the mortality caused in either population (P value = 0.76). However killing of both nematode strains did occur (Figure: 3.12. and 3.13.). Killing of *C. elegans* N2 due to *P. aeruginosa* PA01 began at 72 hours. Conversely the death of *C. elegans phm*-2 began earlier at 24 hours. At 96 hours the percentage mortality of *C. elegans* N2 feeding on *P. aeruginosa* PA01 was 40 % with a calculated TD_{50} value of 99 hours ± 3.39 standard error, while the percentage mortality of *C. elegans phm*-2 under the same experimental conditions was 40.45 % with a TD_{50} value of 104 hours ± 11.51 standard error. This further indicates that there was no difference in the effect of *P. aeruginosa* PA01 on *C. elegans* N2 and *C. elegans phm*-2 in the plate-based mortality assay.

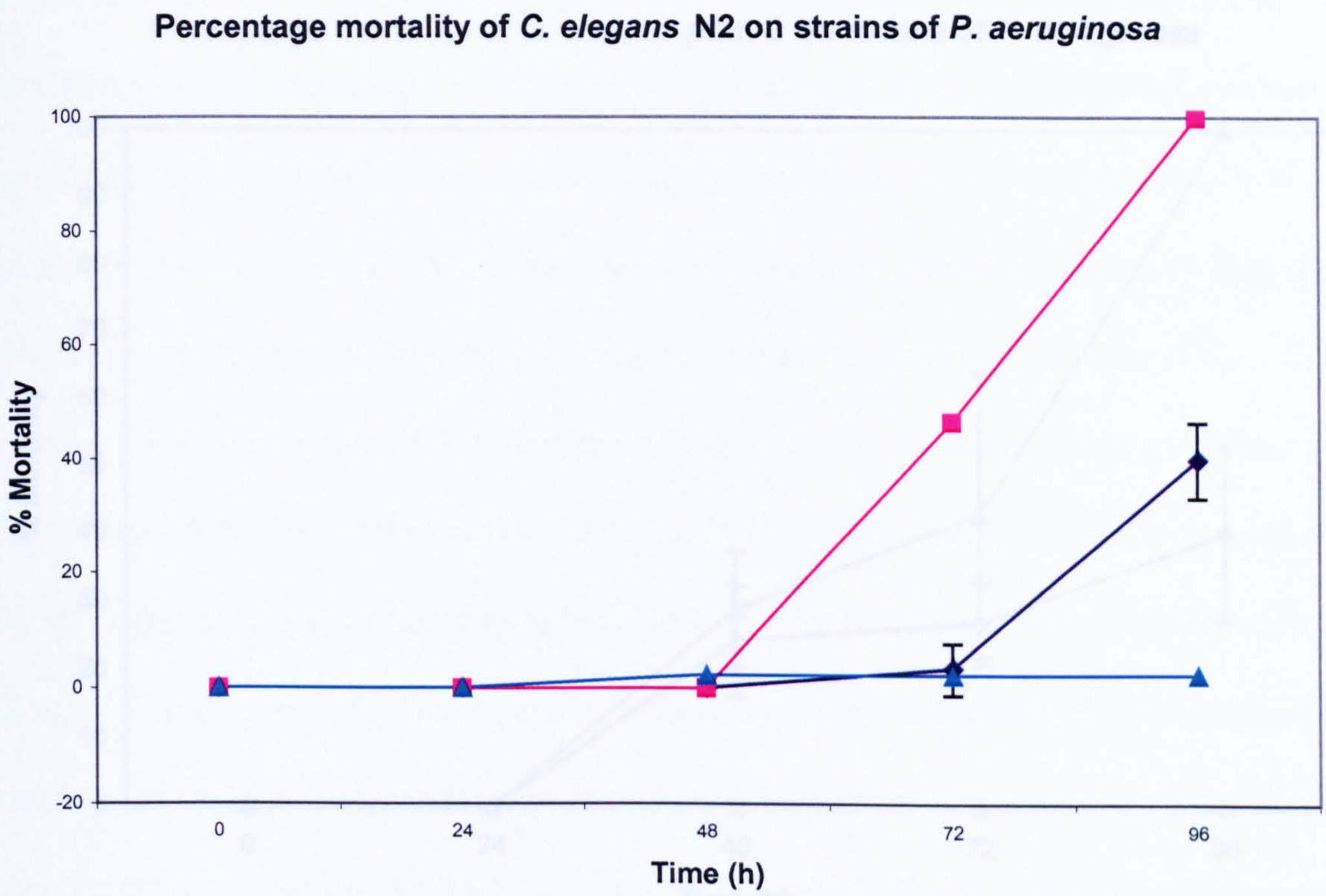


Figure: 3.12. Mortality of *C. elegans* N2 when feeding on *P. aeruginosa* PA01 (◆), *P. aeruginosa* PA14 (■) or *E. coli* OP50 (▲) over 96 hours at 25 °C in the plate-based mortality assay. *P. aeruginosa* PA14 and *E. coli* OP50 were used as controls. Error bars are the standard error of the mean percentage mortality of 3 replicates each containing 10-20 worms.

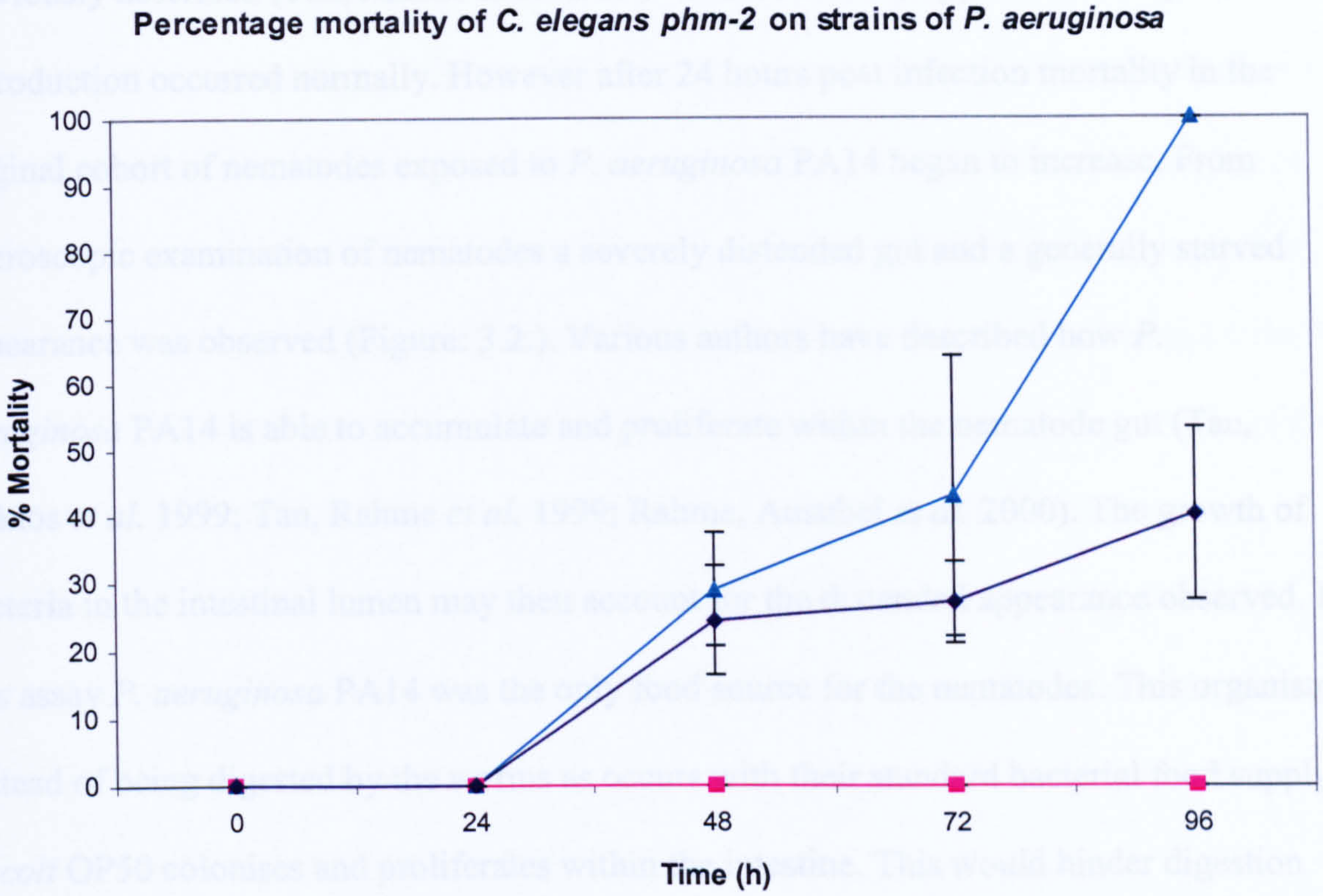


Figure: 3.13. Mortality of *C. elegans phm*-2 when feeding on *P. aeruginosa* PA01 (◆), *P. aeruginosa* PA14 (■) and *E. coli* OP50 (▲) over 96 hours at 25 °C in the plate-based mortality assay. *P. aeruginosa* PA14 and *E. coli* OP50 were used as controls. Error bars are the standard error of the mean percentage mortality of 3 replicates each containing 10-20 worms.

3.7. Discussion.

The results of the experiments reported in this chapter were in accordance with those previously described (Tan, Miklos *et al.* 1999). Worms initially appeared healthy and reproduction occurred normally. However after 24 hours post infection mortality in the original cohort of nematodes exposed to *P. aeruginosa* PA14 began to increase. From microscopic examination of nematodes a severely distended gut and a generally starved appearance was observed (Figure: 3.2.). Various authors have described how *P. aeruginosa* PA14 is able to accumulate and proliferate within the nematode gut (Tan, Miklos *et al.* 1999; Tan, Rahme *et al.* 1999; Rahme, Ausubel *et al.* 2000). The growth of bacteria in the intestinal lumen may then account for the distended appearance observed. In this assay *P. aeruginosa* PA14 was the only food source for the nematodes. This organism, instead of being digested by the worms as occurs with their standard bacterial food supply *E. coli* OP50 colonises and proliferates within the intestine. This would hinder digestion and nutrient up take across the intestine giving the starved appearance that was observed. The starvation would then be a by-product of *Pseudomonas* infection. However the mechanism of killing of *C. elegans* by *P. aeruginosa* PA14 is not completely understood and may occur through an as yet unidentified mechanism. Thus the possibility that the proliferation of *P. aeruginosa* PA14 within the nematode gut is a consequence rather then the cause of killing of *C. elegans* cannot be discounted.

A small increase in the percentage mortality of those worms feeding on *E. coli* OP50 was also noted. This may reflect the normal population dynamic of a growing nematode population under experimental conditions. These observations have been previously reported by Vaupel *et al* (Vaupel, Johnson *et al.* 1994) who showed that the rate of death in

a population of nematodes increases over time. This may then be a factor involved in the increase in mortality in the control population. Moreover it is now thought that as nematodes age they become more susceptible to bacterial infection. Thus in older worms *E. coli* OP50 can become deleterious to health under the standard conditions employed within the killing assay (Garigan 2002). Near death *E. coli* OP50 is seen to accumulate within the nematode pharynx and intestinal tract in a similar fashion to that seen with many of the currently identified pathogens of *C. elegans* (Table: 1.1.). Growth of *C. elegans* on strains of *E. coli,* which are unable to proliferate, has shown that an increase in mean life span of 30-40 % can be achieved (Garigan 2002). This shows that any increase in mortality observed within control populations may be due in part to the natural ageing process of the nematodes. This would degrade the function of the *C. elegans* innate immune system (Section: 1.5.4.5.) allowing bacterial colonisation and an eventual bacterially mediated death of the worm. These data then also suggest that *E. coli* may not be an ideal food source for *C. elegans* as it may not be as benign under the conditions employed in the plate-based mortality assay as is widely accepted.

As an adjunct to the commonly used plate-based mortality assay a novel method of assessment of bacterial virulence was also investigated. An assay developed by Jones and Candido (Jones and Candido 1999) exploited the inhibitory effect of chemicals on nematode feeding. This method has been modified in this study to give a novel method for the evaluation of bacterial virulence towards *C. elegans*. The feeding inhibition assay relies on the ingestion and breakdown of bacteria by nematodes with a resultant reduction in the OD_{550} of the bacterial suspension in which they feed.

P. aeruginosa PA14 caused a slowing of normal nematode feeding rates in suspension when compared to feeding rates on *E. coli* OP50. This decrease in consumption rate when

compared to a suitably non-pathogenic control can then be interpreted as an indication of pathogenicity.

In contrast to the plate-based mortality the feeding inhibition assay is able to supply quantitative data that is not open to operator-induced variation due to differences in the criteria used to identify dead worms. Additionally the feeding inhibition assay also gives a more rapid result compared to other agar pate-based methods. This is shown through comparison of the time to any observable effect in both assay methods. In the plate-based mortality assay, mortality caused by *P. aeruginosa* PA14 is only observable after 48 hours (Figure: 3.1.). However an effect on feeding rate in the feeding inhibition assay can be seen after 24 hours (Figure: 3.6.) therefore allowing a more rapid decision on an organisms pathogenicity to be obtained.

Although the feeding inhibition assay is more rapid than the plate-based mortality assay the mechanism of how an external physical stimulus is converted to a behavioural response in *C. elegans* is unclear and the actual mechanism of feeding inhibition has not been characterised. This then may limit the feeding inhibition assay to the identification of bacterial species that do or do not cause feeding inhibition. It also suggests that unlike the plate-based mortality assay it may not be useful for the identification specific bacterial virulence factors important in mammalian pathogenesis (Miklos, Tan *et al.* 1999; Tan, Rahme *et al.* 1999; Hendrickson, Plotnikova *et al.* 2001; Gan 2002; Tan 2002).

To augment the rapidity of the *C. elegans* model of infection in the plate-based mortality assay and the feeding inhibition assay the mutant *C. elegans phm-2* was investigated. When tested in the plate-based mortality assay killing of the mutant by *P. aeruginosa* PA14 began at 24 hours. Conversely killing of the wildtype *C. elegans* N2 began at 48

hours. This shows that *C. elegans phm-2* is killed more rapidly by *P. aeruginosa* PA14 than the wildtype strain. Furthermore the decrease in time to an observable effect indicates that the mortality of both worm strains is indeed mediated in part by an infectious process. As *C. elegans phm-2* has a defective feeding apparatus an enhanced susceptibility to pathogens that colonise the intestinal tract is expected. Thus the increase in mortality rates observed in the plate-based mortality assay indicates a role for infection in killing of *C. elegans*.

When *C. elegans phm*-2 was assessed in the feeding inhibition assay a decrease in the time to an effect caused by *P. aeruginosa* PA14 was noted when compared to feeding rates of the wildtype *C. elegans* N2. Feeding rates of the mutant were severely reduced by 48 hours (Figure: 3.9.). However, over all feeding rates of *C. elegans phm-2* in the feeding inhibition assay were slower than *C. elegans* N2 on both *E. coli* OP50 and *P. aeruginosa* PA14. Due to the mutation in *C. elegans phm*-2 an increased number of whole bacterial cells are allowed to pass into the gut. The action of the nematode intestine is rapid and the entire gut content is expelled approximately every 45 seconds (Avery 1993). Thus during defecation *C. elegans phm*-2 passes large quantities of intact bacterial cells back into suspension. The increased rate of deposition of whole bacterial cells therefore gives feeding rates that are not truly representative of feeding inhibition by pathogens. This would be in part due to the nature of the mutation as smaller quantities of cells are removed from suspension by the feeding action of the nematode.

As *C. elegans phm*-2 has been shown to be infected more rapidly by *P. aeruginosa* PA14 we reasoned that it might be of use for the investigation of other pathogens not normally capable of causing mortality in the wildtype worm. *P. aeruginosa* PA01 grown on BHI media kills *C. elegans* N2 rapidly through the release of HCN. However when grown on

NGM media mortality is not normally observed (Gallagher and Manoil 2001). To further investigate the increase in sensitivity afforded by *C. elegans phm*-2 the response to *P. aeruginosa* PA01 was assessed using the plate-based mortality assay and the feeding inhibition assay.

When fed on *P. aeruginosa* PA01 in the plate-based mortality assay, mortality of *C. elegans phm*-2 increased at 24 hours in a similar fashion to that seen when *C. elegans phm*-2 fed on *P. aeruginosa* PA14. However killing of the nematodes was incomplete by 96 hours with only 40 % mortality in the population (Figure: 3.13.). Surprisingly *P. aeruginosa* PA01 also killed *C. elegans* N2 in the plate-based mortality assay although the effect was delayed compared to that seen for *C. elegans phm*-2. Killing of *C. elegans* N2 began at 72 hours and reached 40 % by 96 hours (Figure: 3.12.). Calculated TD_{50} values for both mutant and wildtype were also similar. TD_{50} values for mutant and wild type were 104 hours ± 11.51 SE and 99 hours ± 3.39 SE respectively. This indicates no difference in overall killing rate when feeding on *P. aeruginosa* PA01 between the two nematode strains investigated.

These data then suggest an increase in time to an effect of *P. aeruginosa* PA01 infection in *C. elegans phm*-2 as seen with *P. aeruginosa* PA14. This also indicates that the killing observed by *P. aeruginosa* PA01 in the plate-based mortality assay on NGM is mediated at least in part by an infectious process as the mutant is affected earlier when compared to the wildtype strain.

Thus *P. aeruginosa* PA01 is able to cause mortality of both *C. elegans* N2 and the mutant *C. elegans phm*-2 in the plate-based mortality assay at similar rates. Since *P. aeruginosa* PA01 possesses some of the genes implicated in the killing of nematodes by *P. aeruginosa*

PA14 (Stover, Pham *et al.* 2000) it is then possible that under some conditions *P. aeruginosa* PA01 can also display a killing phenotype similar to that of *P. aeruginosa* PA14. This may then be responsible for the increased mortality of both *C. elegans* N2 and *C. elegans phm*-2 in this assay.

Consumption rates of *C. elegans phm*-2 when feeding on pathogen and non-pathogen in the feeding inhibition assay were slower than that of the wildtype strain *C. elegans* N2 (Figures: 3.10. and 3.11.). The slower feeding rates observed for *C. elegans phm*-2 resulting from small changes in OD_{550nm} over time allows a large amount of statistical error to be introduced, hence the lack of statistical significance between feeding rates on either pathogen or non-pathogen at 48 hours. However, the over all trend in this assay was that *P. aeruginosa* PA01 caused a decrease in feeding rates in both *C. elegans phm*-2 and *C. elegans* N2 compared to normal feeding on *E. coli* OP50. This suggests a similar mechanism might be responsible for both feeding inhibition in the feeding inhibition assay and the mortality caused in the plate-based mortality assay as both mutant and wild type were affected, albeit to different extents.

No comparable increase in sensitivity to *P. aeruginosa* PA01 was noted between *C. elegans* N2 and *C. elegans phm*-2 as seen for *P. aeruginosa* PA14. Indeed *P. aeruginosa* PA01appeared to cause a greater decrease in feeding rates in *C. elegans* N2 when compared to *P. aeruginosa* PA14. This again may exemplify a limitation of the feeding inhibition assay in that it can only show that a particular strain is more virulent than another towards *C. elegans*.

Taken together these results indicate that *C. elegans phm*-2 may be used to increase the sensitivity of plate-based assay methods for bacterial virulence. It may also be useful for

the identification of organisms not previously seen to be pathogenic to *C. elegans* under the culture conditions described. Furthermore this would hypothetically allow the expansion of the *C. elegans* model of infection to other bacterial species capable of causing infections in the mammalian host that do not normally cause infections in the wildtype strain *C. elegans* N2. However, the use of *C. elegans phm*-2 in conjunction with the feeding inhibition assay has shown that nematode strains carrying mutations that affect the feeding apparatus may not be suitable for use in this assay due to the generally reduced feeding rates observed.

The investigation of *P. aeruginosa* pathogenesis in the *C. elegans* model has allowed the optimisation and modification of commonly utilised methods for the investigation of bacterial virulence using nematodes. This includes the establishment of standard culture and manipulation techniques essential for the experimental use of *C. elegans*.

Furthermore it has also allowed the development of novel assay methodologies exemplified by the feeding inhibition assay which may provide a further level of sensitivity to this already versatile model

Chapter: 4. Investigation of the effect of the *Burkholderia* genus in *C. elegans*.

4.1. Introduction.

In chapter 3 a nematode model of infection by *P. aeruginosa* was successfully established on solid and in liquid media. However the main aim of this thesis was to investigate the usefulness of the *C. elegans* model for the investigation of *B. pseudomallei* pathogenesis a member of the *Burkholderia* genus. Therefore, to address this a *C. elegans* - *Burkholderia* infection model was established through investigation of a variety of strains from the *Burkholderia* genus.

The *Burkholderia* genus contains a large number of strains, some of which can be pathogenic to humans and other animals. Many of these strains have been assessed for their virulence in both nematode and murine models of infection (O' Quinn, Wiegand et al. 2001; Gan 2002; Kothe 2003). Although these studies have reported a number of interesting findings such as the involvement of particular virulence factors in *C. elegans* pathogenesis, a screen of *Burkholderia* species utilising a representative number stains has so far not been undertaken. Such a screen would allow the clinical relevance of the nematode model for study of strains from the *Burkholderia* genus to be assessed. Comparison of these closely related strains may also reveal common features that would allow a better understanding of the pathogenicity of *B. pseudomallei* in *C. elegans* as a whole. This information could then be utilised to give a further insight into the pathogenesis of this organism in the mammalian host.

4.2. The *B. cepacia* complex.

The effect of a panel of 20 strains from the *B. cepacia* complex comprising of a variety of clinical and environmental isolates was investigated (Table: 4.1.). These represented *B. cepacia*, *Burkholderia multivorans*, *Burkholderia cenocepacia sp. nov*, *Burkholderia stabilis* and *Burkholderia vietnamiensis* (genomovars I-V respectively).

Each strain was initially investigated in the plate-based mortality assay. Groups of 10-20 L4 stage nematodes were fed on lawns of each strain at 25 °C for a total of 96 hours and the mortality in each population recorded at 24-hour intervals. The percentage mortality in each group of worms at 96 hours was then calculated and compared to the mortality of nematodes on *E. coli* OP50. Any increase in percentage mortality compared to that observed when fed on *E. coli* OP50 would indicate a detrimental effect in the nematode model.

Table: 4.1. *B. cepacia* complex isolates selected for investigation in the *C. elegans* model of infection.

[T]. Type strain for that genomovar.

[1]. American Type Culture Collection.

[2]. Belgium Coordinated Collections of Microorganisms/ Laboratorium Microbiologie Ghent (BCCM/LMG) http://www.belspo.be/bccm.

†. These strains were obtained from Dr. J. Govan. CF Laboratory, Department of Medical Microbiology, University of Edinburgh medical school. Edinburgh. UK.

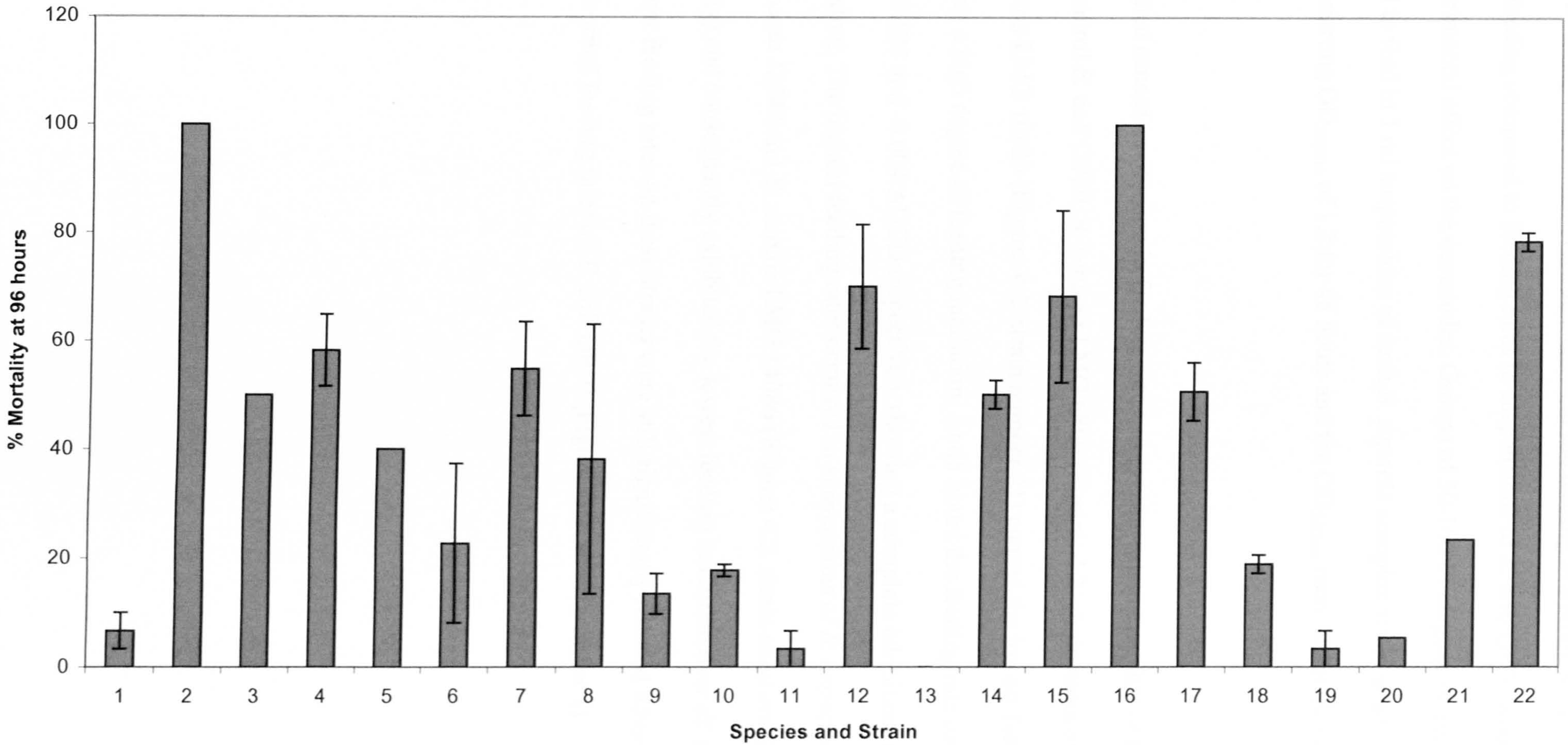
Genomovar	Species Name	Source	Strain Number	Site of isolation
I	*B. cepacia*	ATCC [1]	25416 [T]	Environmental
		ATCC	17759	Environmental
		†	J2540	Environmental
		†	C2970	Clinical
		†	C3159	Clinical
		†	C1963	Clinical
		†	C1964	Clinical
		BCCM/ LMG [2]	6860	Environmental
II	*B. multivorans*	BCCM/ LMG	13010 [T]	Clinical
		ATCC	17616	Environmental
III	*B. cenocepacia*	BCCM/ LMG	16656 [T]	Clinical
		BCCM/ LMG	16654	Clinical
		†	C1394	Clinical
		†	J2956	Clinical
		†	C2836	Clinical
		BCCM/ LMG	18863	Clinical
IV	*B. stabilis*	BCCM/ LMG	14294 [T]	Clinical
		BCCM/ LMG	14086	Clinical
V	*B. vietnamiensis*	BCCM/ LMG	10929 [T]	Environmental
		BCCM/ LMG	18836	Clinical

No single genomovar tested contained more virulent representatives in the plate-based mortality assay than the others tested and only 9 of the strains investigated resulted in 50 % mortality or over at 96 hours. *B. cepacia* (genomovar I) and *B. cenocepacia sp. nov.* (formerly *B. cepacia* complex genomovar III) contained the most virulent strains. However when these were compared the strains tested from *B. cenocepacia sp nov.* resulted in the highest degree of nematode mortality over time. *B. cenocepacia sp. nov.* C2836 (Figure: 4.1. Strain number 16) was the most virulent strain tested causing 100 % mortality at 96 hours. This was equal to that caused by *P. aeruginosa* PA14. Conversely *B. cenocepacia sp. nov.* 18863 (Figure: 4.1. Strain number 13), did not cause nematode death after 96 hours.

The strains tested from the *B. stabilis* group (strains numbers 19 and 20, LMG 14294 and LMG 14086 respectively) had a reduced virulence towards *C. elegans* N2 in the plate-based mortality assay causing only 3.33 % and 5.34 % mortality respectively at 96 hours. Those strains investigated from both the *B. multivorans* group (genomovar II) and *B. vietnamiensis* (genomovar V) had virulent and less virulent representatives. The type strain of each of these genomovars caused the smallest degree of mortality at 96 hours where as the second strain tested caused a large increase in the mortality observed (Figure: 4.1.).

Figure: 4.1. Mortality of *C. elegans* N2 after feeding on lawns of bacteria from genomovars I-V of the *B. cepacia* complex at 25 °C at 96 hours post-infection grown on NGM. Strains tested were *B. cepacia* ATCC 25416 (3), *B. cepacia* C3159 (4), *B. cepacia* ATCC 17759 (5), *B. cepacia* J2540 (6), *B. cepacia* LMG 6860 (7), *B. cepacia* C2970 (8), *B. cepacia* C1963 (9), *B. cepacia* C1964 (10), *B. multivorans* LMG 13010 (11), *B. multivorans* ATCC 17616 (12), *B. cenocepacia* LMG 18863 (13), *B. cenocepacia* J2956 (14), *B. cenocepacia* C1394 (15), *B. cenocepcia* C2836 (16), *B. cenocepcia* LMG 16654 (17), *B. cenocepacia* LMG 16656 (18), *B. stabilis* LMG 14294 (19), *B. stabilis* LMG 14086 (20), *B. vietnamiensis* LMG 10929 (21), *B. vietnamiensis* LMG 18836 (22). The mortality at 96 hours on *P. aeruginosa* PA14 (2) and *E. coli* OP50 (1) has been included as controls. Error bars are the standard error of the mean % mortality at 96 hours of the 3 replicates performed for each strain tested containing between 10-20 L4 stage worms. Some error bars are not visible as the error is too small to display.

Percentage mortality of *C. elegans* N2 at 96 hours when feeding on members of the *B. cepacia* complex.

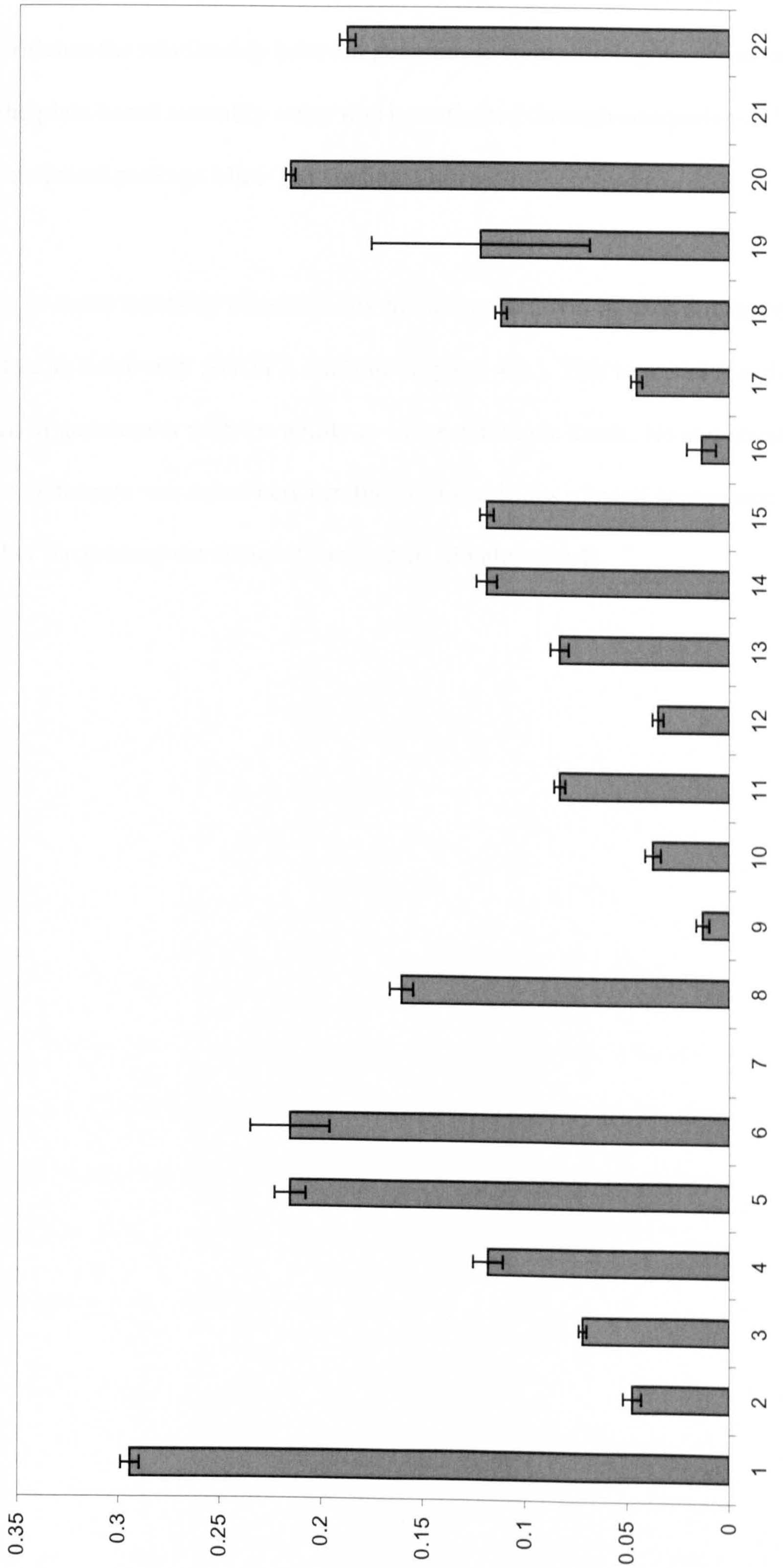


The panel of 20 *B. cepacia* strains were also tested in the feeding inhibition assay. Any reduction in feeding compared to feeding rates in suspensions of *E. coli* OP50 would indicate a detrimental effect on the nematodes. Groups of 50-100 *C. elegans* nematodes were allowed to feed in 1 ml suspensions of each *B. cepacia* complex strain at an approximate starting OD_{550nm} of 1.2 for 48 hours and the OD_{550nm} recorded at 24 hour intervals.

All strains tested caused a reduction in feeding rate to a greater extent than that of the non-pathogenic control *E. coli* OP50. *B. cepacia* LMG 6860 (Figure: 4.2. strain number 6) and *B. vietnamiensis* LMG 10929 (Figure: 4.2. strain number 21) caused the lowest feeding rates indicating a high degree of feeding inhibition. At 48 hours the feeding rate on these strains was - 0.023 and -0.004 AUCD respectively showing a complete inhibition of nematode feeding. The highest feeding rates occurred in suspensions of *B. cepacia* ATCC 17759, *B. cepacia* J2540 and *B. stabilis* LMG 14086 (Figure: 4.2. strain numbers 5, 6 and 20 respectively) and consequently exhibited the lowest feeding inhibition over all at 48 hours. However feeding rates on these strains were still significantly reduced when compared to normal feeding rates on *E. coli* OP50 (Figure. 4.2. strain number 1).

Figure: 4.2. Change in OD_{550nm} at 48 hours of bacterial suspensions of strains from genomovars I-V of the *B. cepacia* complex at 25 °C. Each 1 ml sample of bacterial suspension in K-medium had a starting OD_{550nm} of approximately 1.2. Strains tested were *B. cepacia* ATCC 25416 (3), *B. cepacia* C3159 (4), *B. cepacia* ATCC 17759 (5), *B. cepacia* J2540 (6), *B. cepacia* LMG 6860 (7), *B. cepacia* C2970 (8), *B. cepacia* C1963 (9), *B. cepacia* C1964 (10), *B. multivorans* LMG 13010 (11), *B. multivorans* ATCC 17616 (12), *B. cenocepacia* LMG 18863 (13), *B. cenocepacia* J2956 (14), *B. cenocepacia* C1394 (15), *B. cenocepcia* C2836 (16), *B. cenocepcia* LMG 16654 (17), *B. cenocepacia* LMG 16656 (18), *B. stabilis* LMG 14294 (19), *B. stabilis* LMG 14086 (20), *B. vietnamiensis* LMG 10929 (21), *B. vietnamiensis* LMG 18836 (22). The feeding rates at 48 hours on *P. aeruginosa* PA14 (2) and *E. coli* OP50 (1) were included as controls. Error bars are the standard error of the mean AUCD for the 8 replicates performed for each strain tested containing 50-100 L4 stage worms.

Feeding rates of *C. elegans* N2 on *B. cepacia* complex isolates at 48 h



In an attempt to determine the relevance of the *C. elegans* model to the study of the *B. cepacia* virulence the relationship between genomovar status, biological origin and the effect in the plate-based mortality assay was investigated through comparison of the data using the statistical package Mini-Tab version 13.0.

The ability to cause mortality of nematodes and the genomovar of each strain were compared using a one-way ANOVA analysis (Figure: 4.3.). This revealed that there was no association of genomovar with the ability to cause nematode death. No statistically significant difference was noted between the killing abilities of each genomovar when compared to the genomovar status of each strain (P value = 0.4).

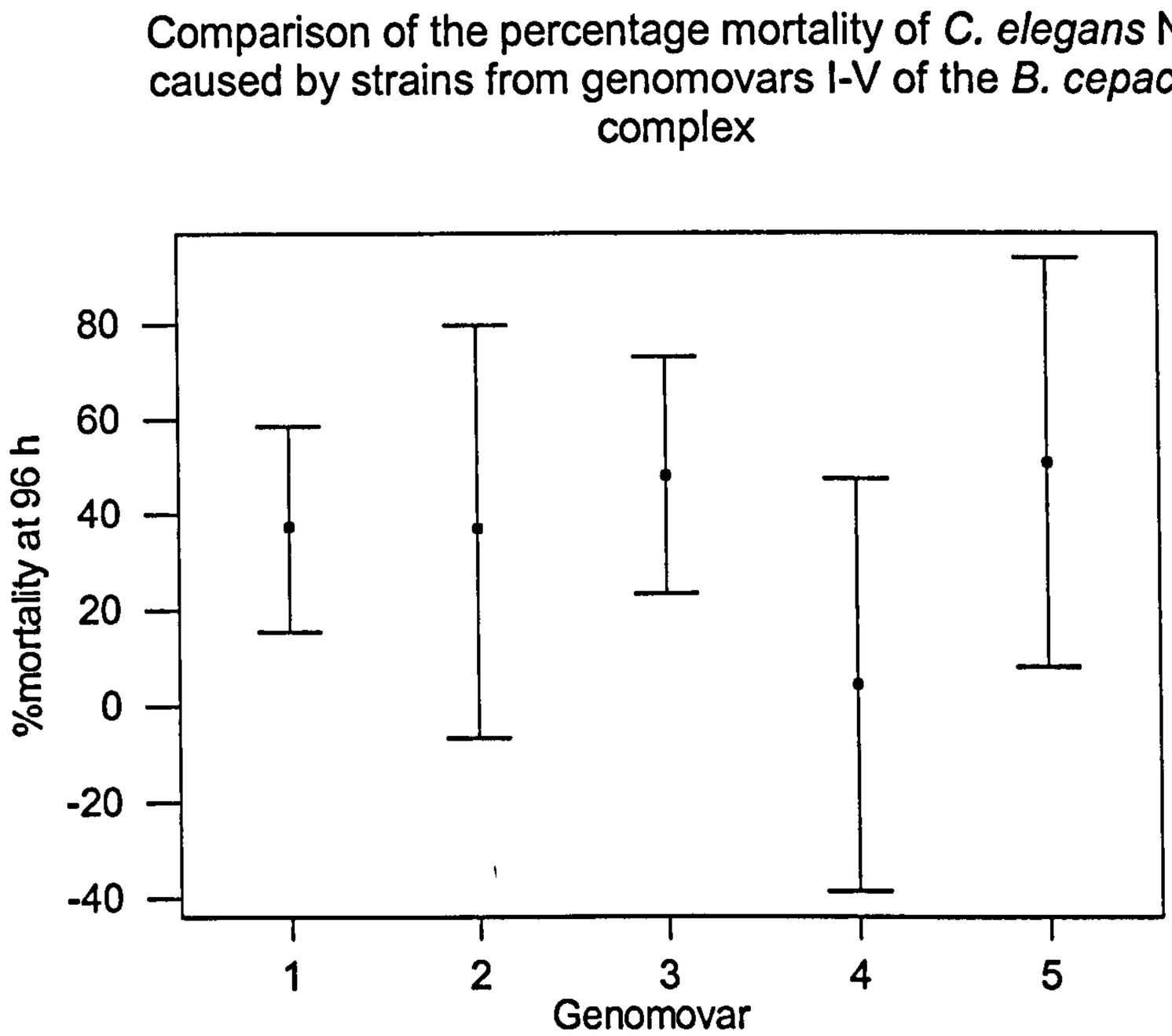


Figure: 4.3. Comparison of the percentage mortality observed in populations of *C. elegans* N2 at 96 hours when feeding on strains from genomovars I-V of the *B. cepacia* complex at 25 °C and the genomovar status of each group. Filled squares show the mean percentage mortality for that genomovar. Bars denote the individual 95% confidence intervals based on pooled standard deviations from each genomovar.

To show if the origin of the infecting strain could influence the effect in either assay a comparison was also performed between the origin of the infecting strain and the effect in both the plate-based mortality assay and the feeding inhibition assay using a two-sample t-test. This indicated that there was no statistically significant difference between the effect of strains from either environmental or clinical sources in the plate based mortality assay or the feeding inhibition assay with P values of 0.5 and 0.7 respectively.

4.3. Aberrant movement of *C. elegans* induced by members of the *B. cepacia* complex.

During screening of the 20 strains from the *B. cepacia* complex an unusual phenotype was observed that was induced by some of the strains tested. This phenotype was characterised by an increased side to side flexion of the body which formed trails in the bacterial lawn that were distinct from the normal sinusoidal pattern made by worms fed on either *P. aeruginosa* PA14 or *E. coli* OP50. In some cases this resulted in a loss in the ability of the nematodes to effectively translocate across the bacterial lawn (Figure: 4.4.).

Strains causing aberrant movement included *B. cepacia* strains ATCC 25416, ATCC 17759, J2540, and C2970; *B. cenocepacia sp. nov* strains J2956, LMG 16654 (J415) and C2836. *B. vietnamiensis* strains LMG 10929 and LMG 18836 also caused difficulties in nematode translocation across the bacterial lawn. None of the strains tested from either *B. multivorans* or *B. stabilis* caused aberrant movement after 96 hours at 25 °C.

When feeding on the above strains the aberrant movement phenotype appeared as early as 24 hours post-exposure and persisted throughout the experiment with varying degrees of severity. *B. cenocepacia sp. nov* strains J2956, LMG 16654 and *B. vietnamiensis* LMG 10929 caused the most severe phenotype. Microscopic examination showed that worms feeding on these strains became completely immobilised. Furthermore no particular larval stage displayed the aberrant movement phenotype more than any other in the growing population as all larval stages appeared to be affected. However the larger L4, young adult and adult stages were observed to have less difficulty in translocation across the bacterial lawn.

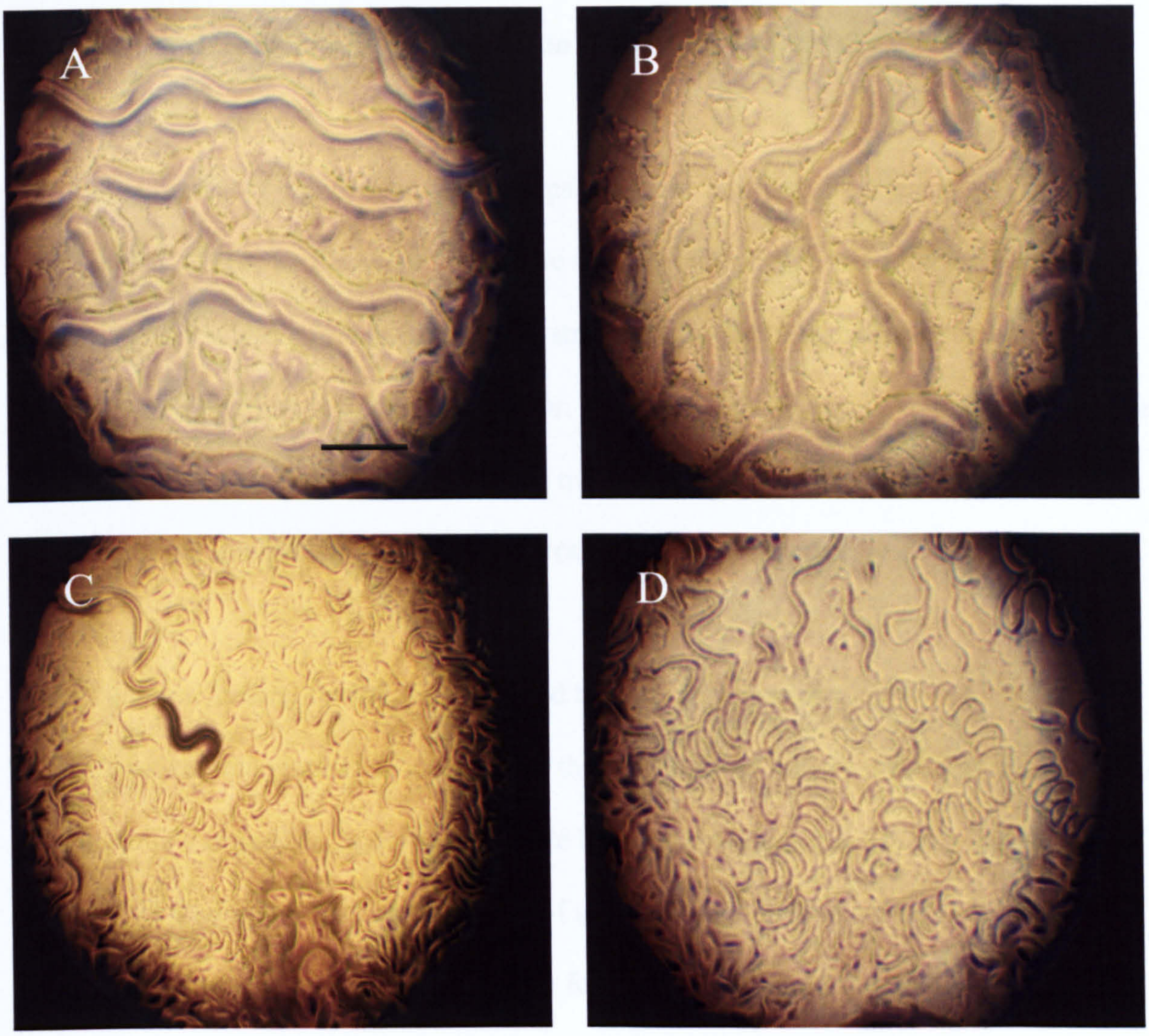


Figure: 4.4. Photomicrographs of NGM agar plates seeded with bacterial cells cultured at 37 °C on which *C. elegans* N2 has been allowed to feed for 72 hours at 25 °C. A; normal trails on *E. coli* OP50 after 72 hours. B; normal trials left on the pathogenic *P. aeruginosa* PA14 after 72 hours. C; a *C. elegans* N2 nematode displaying a severe aberrant movement phenotype and partially immobilised on a lawn of *B. cepacia* ATCC 17759 after 72 hours. D; evidence of severe aberrant movement seen after 72 hours feeding on *B. vietnamiensis* LMG 18836. Scale bar represents 250 μm.

4.4. Effect of *B. cenocepacia sp. nov.* strain LMG 16654 in the *C. elegans* model.

During the course of the investigation one particular strain from *B. cenocepacia sp. nov.* , LMG 16654 (J415) was observed to induce a second phenotype in addition to aberrant movement that was distinct from the other strains tested. J415 is the index strain for cepacia syndrome in the CF community (Dr. J. Govan, Pers. Comm.). It has both the cable pilin (Cbl^+) and *B. cepacia* epidemic strain marker (BCESM) genetic elements, which are linked with the transmission of epidemic strains (Clode, Kaufmann *et al.* 2000).

After 24 hours feeding on J415 it was noted that the organism as well as inducing the aberrant movement phenotype also caused the formation of adherent structures on the head of nematode that further hindered nematode locomotion (Figure: 4.5.). In populations of *C. elegans* N2 feeding on J415 the presence of this "nose-glob" phenotype, as shown by the percentage of worms on which a nose glob formed (%NGF) peaked at 24 hours and subsequently decreased in prevalence over the course of the experiment. This continued until 96 hours after which no nematodes could be observed to retain any nose-globs and normal locomotive ability had returned to those previously affected. (Figure: 4.6.).

Figure: 4.5. Photomicrograph of *C. elegans* N2 with a nose-glob after feeding for 24 hours at 25 °C on *B. cenocepacia sp.nov.* J415 grown on NGM. The arrow shows the accumulation at the head of the nematode that impedes locomotion. Scale bar = 200 μm

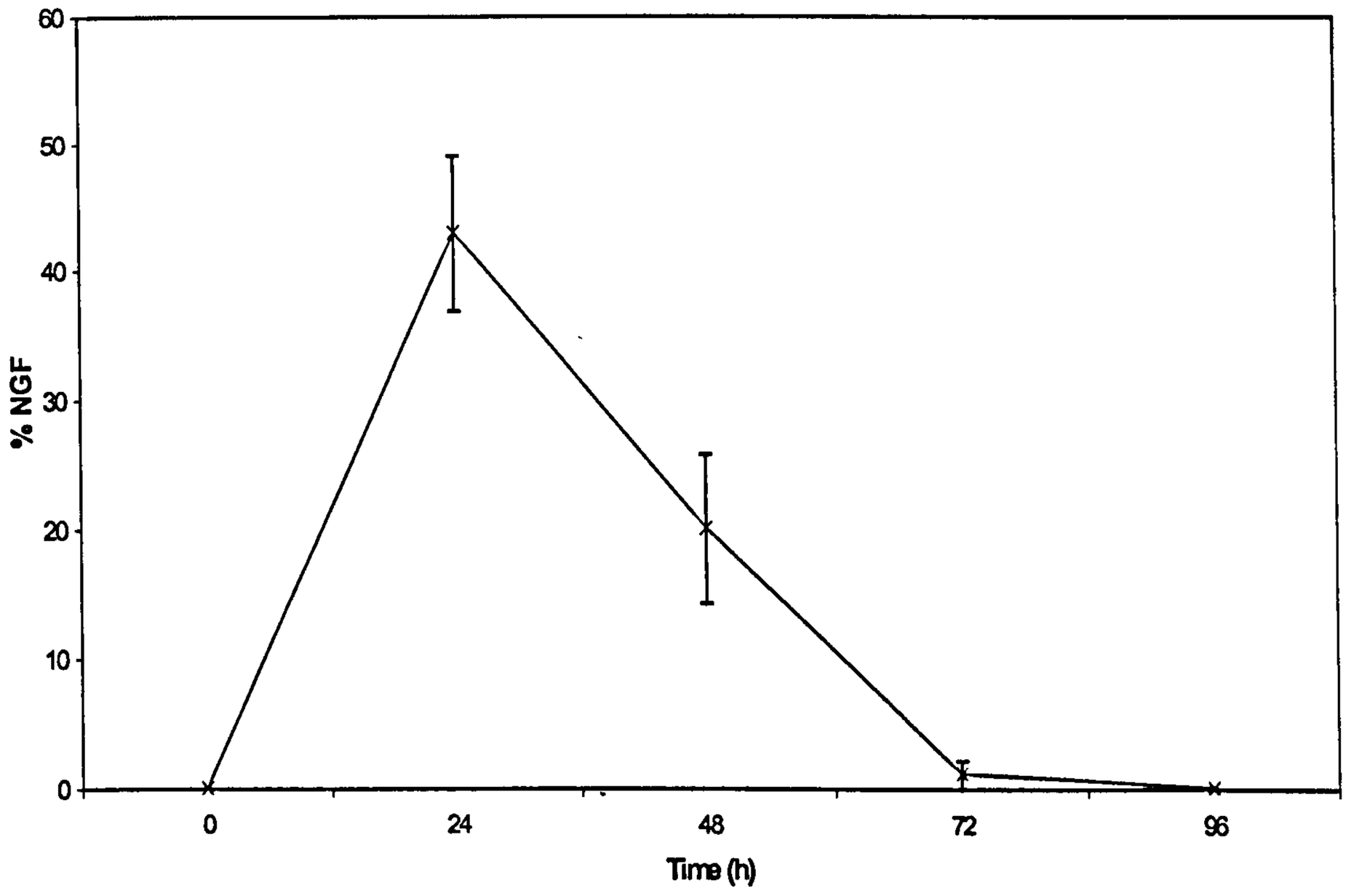


Figure: 4.6. Occurrence of the nose-glob phenotype in populations of *C. elegans* N2 when feeding on *B. cenocepacia sp. nov* J415 grown on NGM. Worms were fed at 25 °C for 96 hours and observed through daily microscopic examination of plates. Error bars are the standard error of mean %NGF of the 9 replicates performed each containing 10-20 L4 stage worms.

4.4.1. Affect of cuticle structure on formation of nose-globs.

The *C. elegans* mutants *srf*-2, *srf*-3 and *srf*-5 posses mutations that effect the cells which form the hypodermis. These cells are in turn, responsible for the secretion of the highly impermeable cuticle (Politz 1990). Although wildtype in appearance, these mutants have an ability to bind lectins such as wheat germ agglutinin which is different to that observed in the wildtype strain *C. elegans* N2. Surface componénts normally hidden in the adult N2 strain are unmasked, giving a different lectin binding profile and surface structure for each mutant (Politz 1987; Politz 1990; Link 1992).

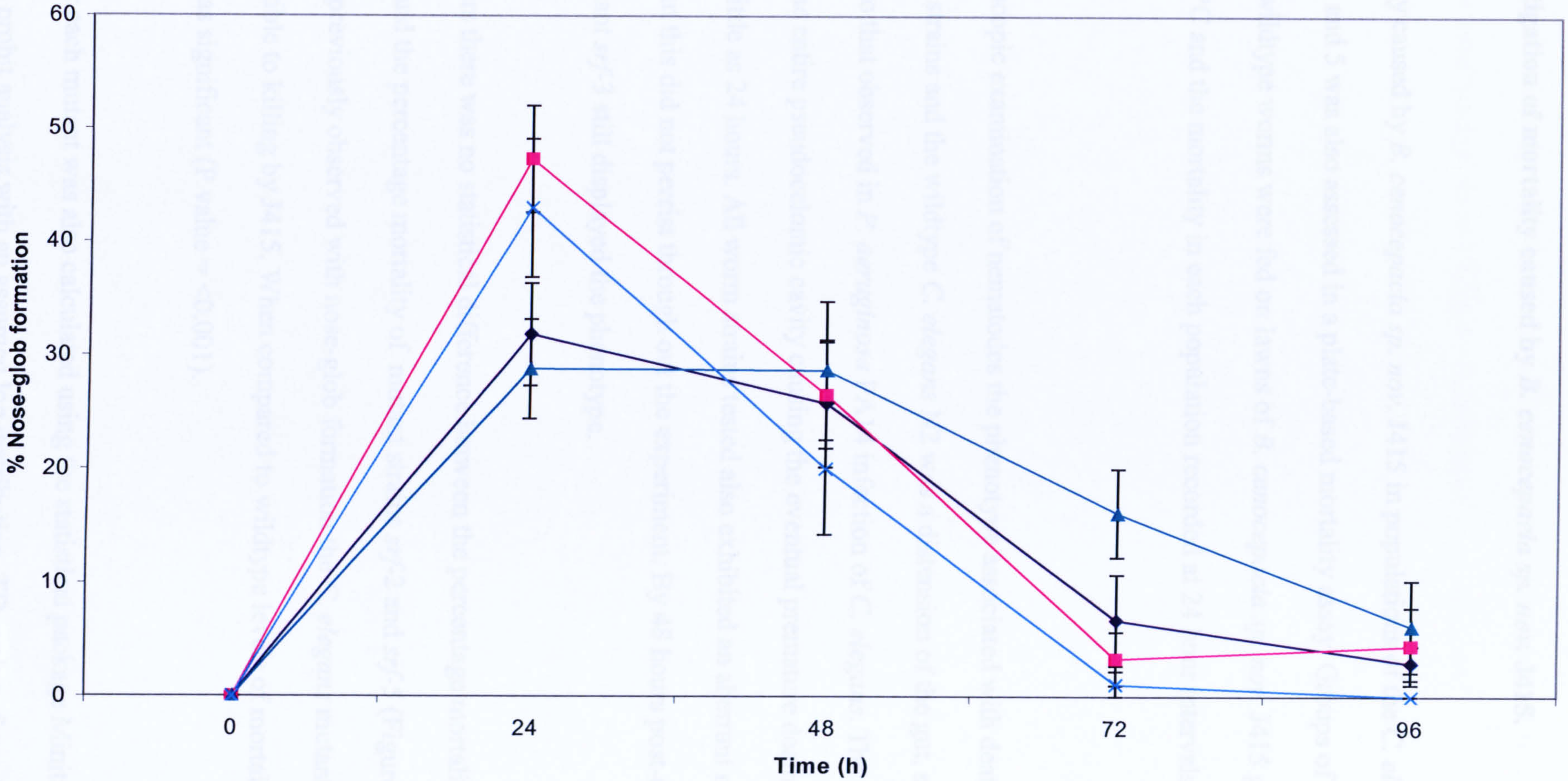
These mutants have previously been shown to be refractory to infection by a number of fungal and bacterial species, which are pathogeic for wildtype *C. elegans* N2 (De Gives 1999). It has also recently been demonstrated that they are resistant to the blockage and nose-glob formation induced by *Y. pseudotuberculosis* (Section: 1.5.4.6). The differences in cuticle structure displayed by these mutants allow resistance to the accumulation of *Y. pseudotuberculosis* cells in the form of biofilms on the cuticular surfaces of nematode body. These impede normal nematode feeding and locomotion and are a factor in causing the precocious death of the worm (Joshua 2003).

As the nose-glob phenotype observed in *C. elegans* N2 when feeding on *B. cenocepacia sp. nov.* J415 was similar to that observed when feeding on *Yersinia* species the susceptibility of *srf*-2, 3 and 5 to the nose-glob formation induced by *B. cenocepacia sp. nov.* J415 was investigated using a modified plate-based mortality assay. This would show if a similar mechanism of nose-glob formation occurring between the two bacterial species.

Groups of 10-20 *C. elegans srf* 2, 3 and 5 and N2 wildtype worms were allowed to feed on lawns of *B. cenocepacia sp. nov.* J415 for a total of 96 hours and the number of worms which were observed to possess nose-globs was recorded at 24 hour intervals. All *srf*-mutant strains tested displayed the adherent nose-glob phenotype at 24 hours (Figure: 4.7.). When compared to *C. elegans* N2 there was no significant difference in the ability to cause nose-globs between any of the mutant strains tested. However at 24 hours the mutant *srf*-5 appeared to be most resistant to nose-glob formation with only 28 % NGF. Nose-globs were seen to persist longer in this strain than the other strains tested. The mutant *srf*-3 was the most susceptible strain over all with 47 % NGF at 24 hours (Figure: 4.7.).

Figure: 4.7. Nose-glob formation in the *C. elegans srf*-mutants 2, 3 and 5 on *B. cenocepacia sp. nov.* J415 grown on NGM. Worms were fed at 25 °C over 96 hours and observed by microscopy. Nose-glob formation in *C. elegans* N2 was used as a control. ◆ - nose-glob formation in *C. elegans srf*-2, ■ - *C. elegans srf*-3, ▲ - *C. elegans srf*-5 and finally × - in *C. elegans* N2. Error bars are the standard error of the mean % NGF for the 9 replicates performed for each strain each containing 10-20 L4 stage worms.

Nose-glob formation in the *C. elegans srf* mutants 2, 3 and 5 on the *B. cenocepacia sp. nov.* strain J415.



4.4.2. Investigation of mortality caused by *B. cenocepacia sp. nov.* J415.

The mortality caused by *B. cenocepacia sp. nov.* J415 in populations of the *C. elegans srf* mutants 2, 3 and 5 was also assessed in a plate-based mortality assay. Groups of 10-20 mutant and wildtype worms were fed on lawns of *B. cenocepacia sp. nov.* J415 grown on NGM at 25 °C and the mortality in each population recorded at 24 hour intervals over a 96 hour period.

From microscopic examination of nematodes the phenotype associated with death of the both mutant strains and the wildtype *C. elegans* N2 was a distension of the gut, similar in appearance to that observed in *P. aeruginosa* PA14 infection of *C. elegans*. The gut was seen to fill the entire pseudocelomic cavity causing the eventual premature death of the worm in as little as 24 hours. All worm strains tested also exhibited an aberrant movement phenotype but this did not persist through out the experiment. By 48 hours post-exposure only the mutant *srf*-3 still displayed the phenotype.

After 96 hours there was no statistical difference between the percentage mortality of *C. elegans* N2 and the percentage mortality of mutant strains *srf*-2 and *srf*-5 (Figure: 4.8.). However as previously observed with nose-glob formation the *C. elegans* mutant *srf*-3 was most susceptible to killing by J415. When compared to wildtype levels of mortality the difference was significant (P value = <0.001).

The TD_{50} for each mutant was also calculated using the statistical package Minitab version 13.1 through probit analysis with an assumed log distribution. TD_{50}values for *srf*-2, 3, 5 mutant nematodes and the wild type *C. elegans* N2 were 104 hours +/- 4.2 standard error,

74 hours +/- 3.2 standard error, 113 hours +/- 6.4 standard error and 111 hours +/- 7.4 standard error respectively. The decrease in TD_{50} shows that of the mutant strains tested *srf*-3 was the most susceptible to killing by *B. cenocepacia sp. nov.* J415.

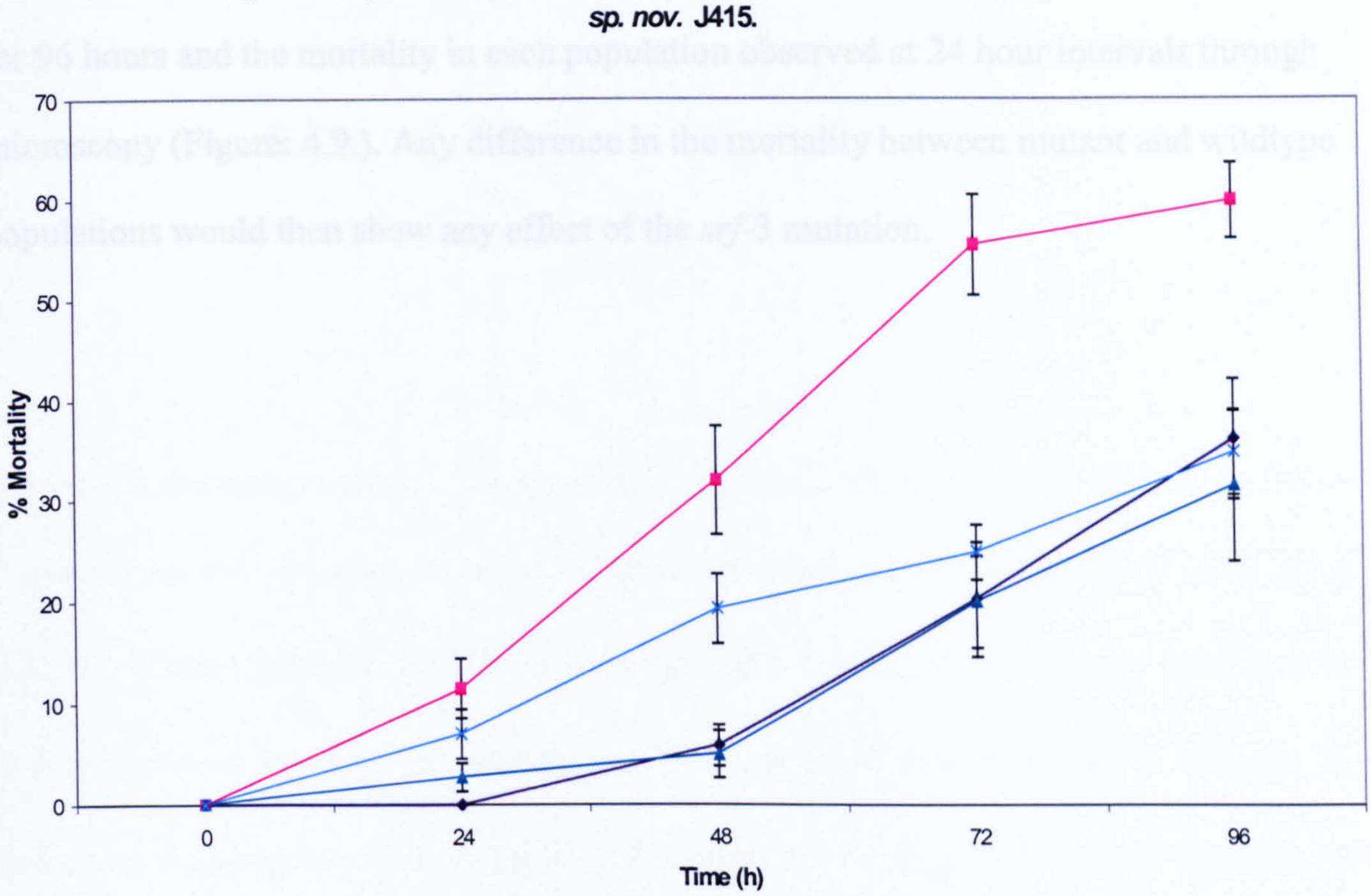
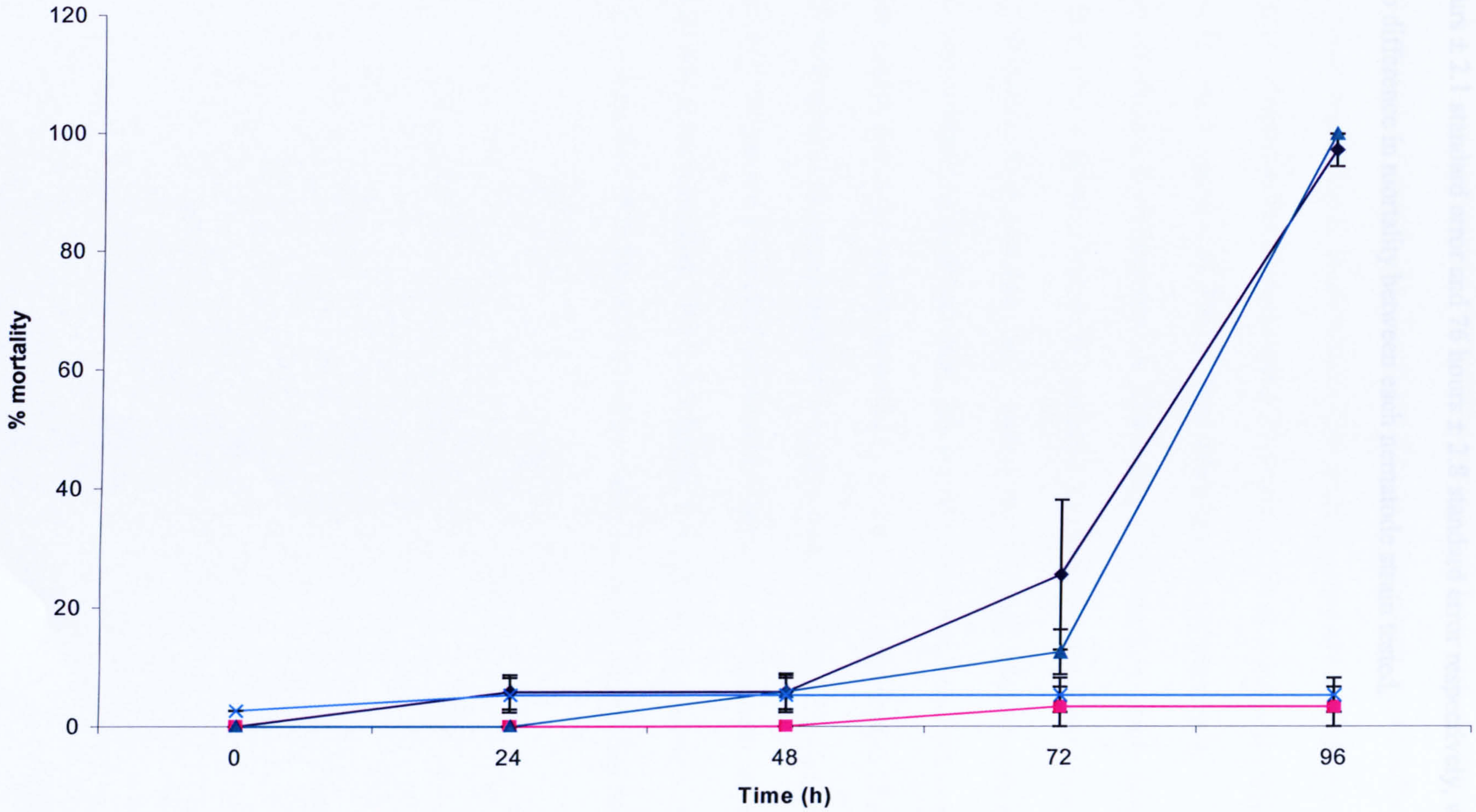


Figure: 4.8. Mortality of *C. elegans srf* mutants 2 (◆), 3 (■), 5 (▲) or the wildtype N2 (×) when feeding on the *B. cenocepacia sp. nov.* J415 grown on NGM. Nematodes were fed at 25 °C for 96 hours. Error bars are the standard error of the mean of 9 replicates performed for each strain each containing 10-20 L4 stage worms.

The mutant *C. elegans srf-* 3 was the only mutant that displayed a significant increase in percentage mortality on *B. cenocepacia sp. nov.* J415 when compared to *C. elegans* N2, in the plate-based mortality assay. The possibility of the mutation in *C. elegans srf*-3 causing a general increase in the susceptibility of this strain to bacterial infection was investigated. Groups of 10-15 *C. elegans srf*-3 or *C. elegans* N2 worms were fed on *P. aeruginosa* PA14 for 96 hours and the mortality in each population observed at 24 hour intervals through microscopy (Figure: 4.9.). Any difference in the mortality between mutant and wildtype populations would then show any effect of the *srf*-3 mutation.

Figure: 4.9. Mortality rates of *C. elegans* N2 and the *C. elegans* mutant *srf*-3 when fed on *P. aeruginosa* PA14 grown on NGM. Worms were allowed to feed for a total of 96 hours at 25 °C. Worms fed on *E. coli* OP50 were used as a control. ◆ - *C. elegans srf*-3 feeding on *P. aeruginosa* PA14, ■ - *C. elegans srf*-3 feeding on *E. coli* OP50, ▲ - *C. elegans* N2 feeding on *P. aeruginosa* PA14 and × - *C. elegans* N2 feeding on *E. coli* OP50. Error bars are the standard error of the mean of 3 replicates performed for each condition, each containing 10-15 L4 stage worms.

Comparison of mortality rates between *C. elegans* N2 and the *C. elegans* mutant *srf*-3 when feeding on *P. aeruginosa* PA14.



After 96 hours there was no significant difference observed between mortality rates of the mutant *C. elegans srf*-3 or *C. elegans* N2 when fed on *P. aeruginosa* PA14 (P value = 0.3). TD_{50} values for *C. elegans* N2 and *C. elegans srf*-3 when fed on *P. aeruginosa* PA14 were 79 hours ± 2.1 standard error and 76 hours ± 2.8 standard error respectively, which indicates no difference in mortality between each nematode strain tested.

4.5. Comparison of *B. pseudomallei* and *B. thailandensis* to the *B. cepacia* complex.

B. pseudomallei, *B. thailandensis* and those strains comprising the *B. cepacia* complex are related phenotypically and genetically (Kanai and Kondo 1994). Furthermore different strains of *B. pseudomallei* and *B. thailandensis* have been reported to have different abilities to kill *C. elegans* as has been observed for strains of the *B. cepacia* complex in this study (O' Quinn, Wiegand *et al.* 2001). Thus, strain-specific differences in pathogenicity are evident in all three species. The ability to discern these strain specific differences in virulence between isolates is important for the fulfilment of the aims of this study. If any differences in *B. pseudomallei* virulence observed in *C. elegans* correspond to those seen in mammalian models of infection, this would reveal the clinical relevance of the *C. elegans* model. Hence the nematode would be found to be a good model of *B. pseudomallei* pathogenesis in the mammalian host. However, to begin the investigation of *B. pseudomallei* pathogenesis a model of nematode killing under containment, at biological safety level III was first established. This was achieved through the investigation of the wildtype *B. pseudomallei* 576 in the plate-based mortality assay and the feeding inhibition assay.

4.5.1. Killing of *C. elegans* N2 by *B. pseudomallei* 576.

Groups of 10-20 L4 stage *C. elegans* N2 worms were fed on lawns of *B. pseudomallei* 576 or *E. coli* strain OP50 grown on NGM. The percentage mortality in each population was then recorded at 24 hour intervals over 96 hours at 25 °C. After 96 hours there was no mortality observed when nematodes had fed on *E. coli* OP50. However when fed on *B. pseudomallei* 576 there was 100% mortality of nematodes by 48 hours (Figure: 4.10.). The TD_{50} when feeding on *B. pseudomallei* 576 was 28 hours ± 1.3 standard error. The difference in mortality caused between *B. pseudomallei* 576 and *E. coli* OP50 was highly significant with a P value of <0.001 at 24 hours.

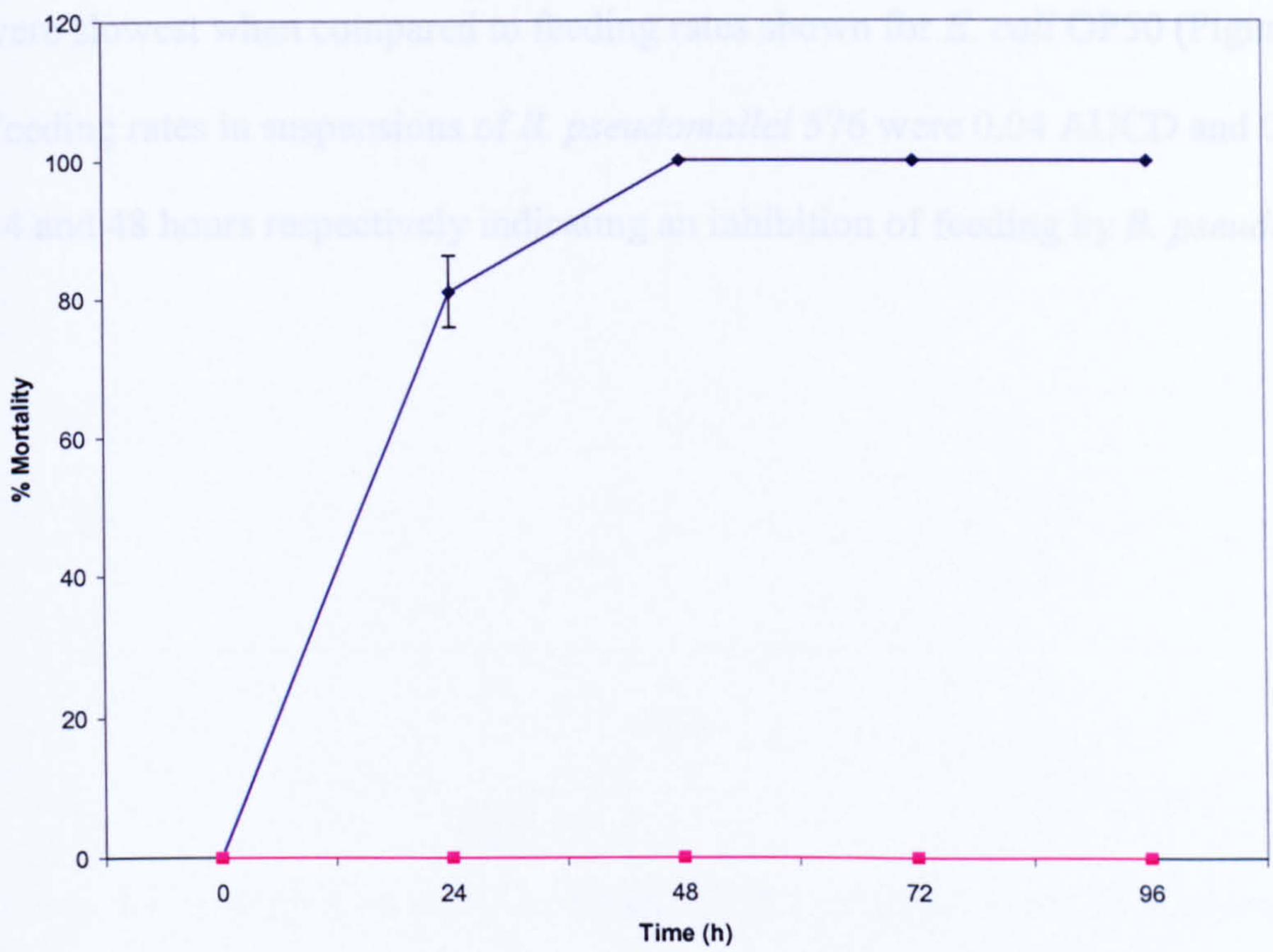


Figure: 4.10. Mortality of *C. elegans* N2 in the plate-based mortality assay at 25 °C when fed on overnight cultures of *B. pseudomallei* 576 (◆) grown on NGM. Mortality on *E. coli* OP50 (■) at 25 °C was used as a control. Error bars are the standard error of the mean of the 9 replicates performed each containing 10-20 L4 stage nematodes.

Feeding inhibition of *C. elegans* N2 by *B. pseudomallei* 576 was also investigated in the feeding inhibition assay. Groups of 50-100 nematodes were allowed to feed in suspensions of either *B. pseudomallei* 576 or *E. coli* OP50 at 25 °C and the change in OD_{550nm} over time recorded. The rate of feeding in *E. coli* OP50 increased from 0.160 AUCD at 24 hours to 0.26 AUCD at 48 hours. However after 48 hours feeding rates in *B. pseudomallei* 576 were slowest when compared to feeding rates shown for *E. coli* OP50 (Figure: 4.11.). Feeding rates in suspensions of *B. pseudomallei* 576 were 0.04 AUCD and 0.04 AUCD at 24 and 48 hours respectively indicating an inhibition of feeding by *B. pseudomallei* 576.

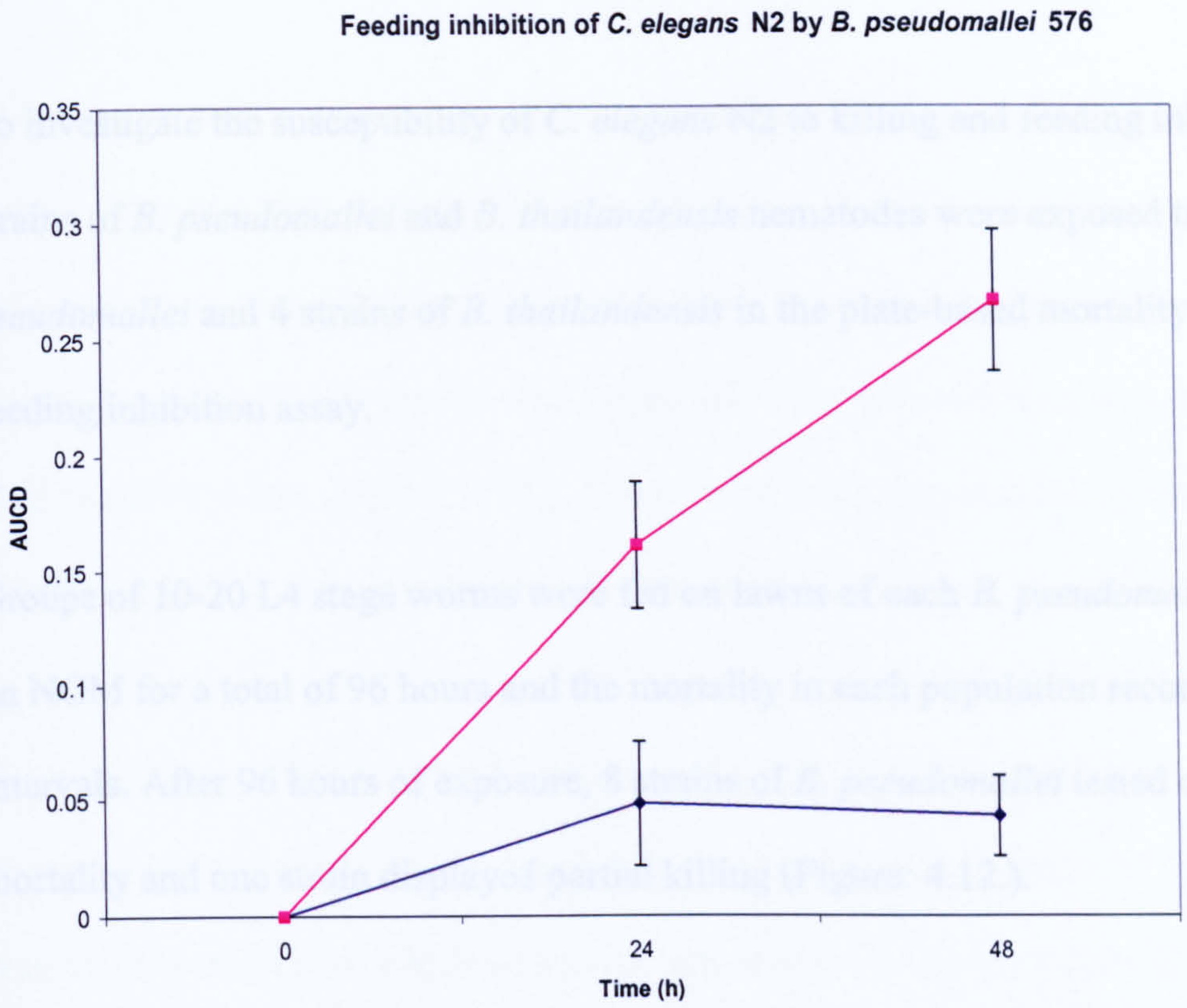


Figure: 4.11. Feeding rates of *C. elegans* N2 in 1 ml suspensions of *B. pseudomallei* 576 (◆) in K-medium at 25 °C over 48 hours. Feeding in *E. coli* OP50 (■) suspensions at 25 °C was used as a control. Error bars are the standard error of the mean of 40 replicates performed for each organism each containing 50-100 L4 stage nematodes.

4.5.2. Investigation of *B. pseudomallei* and *B. thailandensis* strains.

To investigate the susceptibility of *C. elegans* N2 to killing and feeding inhibition by strains of *B. pseudomallei* and *B. thailandensis* nematodes were exposed to 9 strains of *B. pseudomallei* and 4 strains of *B. thailandensis* in the plate-based mortality assay and the feeding inhibition assay.

Groups of 10-20 L4 stage worms were fed on lawns of each *B. pseudomallei* strain grown on NGM for a total of 96 hours and the mortality in each population recorded at 24 hour intervals. After 96 hours of exposure, 8 strains of *B. pseudomallei* tested caused 100% mortality and one strain displayed partial killing (Figure: 4.12.).

B. pseudomallei K96243 caused 100 % mortality at 96 hours in the plate-based mortality assay. However at 24 hours the difference in killing rates between *B. pseudomallei* 576 and strain K96243 was highly significant (P = <0.001). The calculated TD_{50} value was 35 hours ± 1.5 standard error. This was in excess of that calculated for *B. pseudomallei* 576 which was 28 hours ± 1.3 standard error. All of the other fully virulent strains assessed had TD_{50} values of less than 24 hours. This indicates a difference in virulence in the *C. elegans* model between *B. pseudomallei* K96243 and *B. pseudomallei* 576.

B. pseudomallei 42 caused 41 % mortality of *C. elegans* N2 at 96 hours post exposure in the plate-based mortality assay. When compared to killing by *B. pseudomallei* 576 the difference in killing rate was statistically significant with a P value of <0.001. The TD_{50} values of strain 42 and 576 were 107 hours ± 8.1 standard error and 28 hours ± 1.3

standard error respectively. This further indicates a difference in virulence between the strains tested.

When the same *B. pseudomallei* strains were assessed in the feeding inhibition assay 8 strains induced a high degree of feeding inhibition. The OD_{550nm} of the bacterial suspension was also often seen to increase by 48 hours indicating growth of the organism within the assay. This is represented graphically after processing of data as feeding rates with negative values (Figure: 4.13.). The level of inhibition was also greater than that seen for many of the *B. cepacia* complex strains investigated.

In the feeding inhibition assay *B. pseudomallei* ATCC 23343 was the only strain that failed to significantly reduce feeding. The feeding rate on *B. pseudomallei* ATCC 23343 at 48 hours was 0.19 AUCD, which was 59 % of that seen for *E. coli* OP50 (0.32 AUCD at 48 hours). When compared to rates of feeding on *B. pseudomallei* 576 the difference in feeding rate on *B. pseudomallei* ATCC 23343 at 48 hours was highly significant (P value = <0.001).

As a more stringent statistical comparison a one-way ANOVA with a Dunnetts comparison was carried out. This allows feeding rates on one strain to be compared to feeding rates on the others. The comparison revealed that feeding rates on *B. pseudomallei* ATCC 23343 was not significantly different to those on *B. pseudomallei* 576 or *B. pseudomallei* 52 (P=<0.05). However significant differences in feeding rate did exist between *B. pseudomallei* ATCC 23343 and the remaining strains tested indicating strain dependant differences in the effect on feeding rate in the feeding inhibition assay.

Figure: 4.12. Mortality of *C. elegans* N2 at 25 °C over 96 hours when fed on wildtype strains of *B. pseudomallei* grown on NGM. Strains tested were *B. pseudomallei* strain 4845 (◆), strain 52 (■), strain 204 (▲), ATCC 23343 (✶), strain BRI (●), strain 42 (▮), strain 2889 (×) or strain K96243 (◆). The mortality caused by each strain was compared to *B. pseudomallei* 576 (-) and *E. coli* OP50 (–), which were chosen to serve as controls. Error bars are the standard error of the mean AUCD of 3 replicates performed for each strain each containing 50-100 L4 stage worms.

Percentage mortalty of *C. elegans* N2 when feeding on different strains of *B. pseudomallei*.

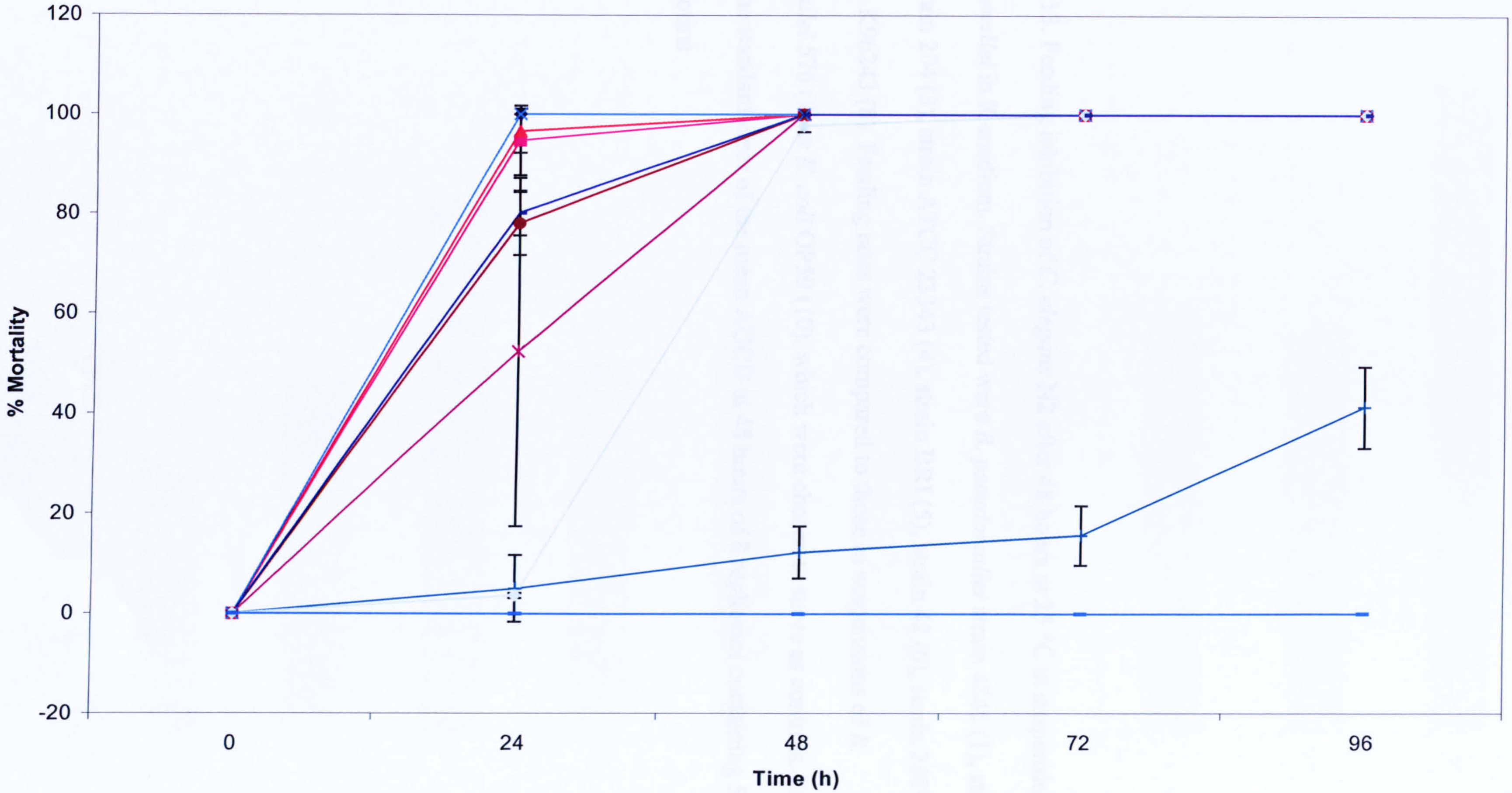
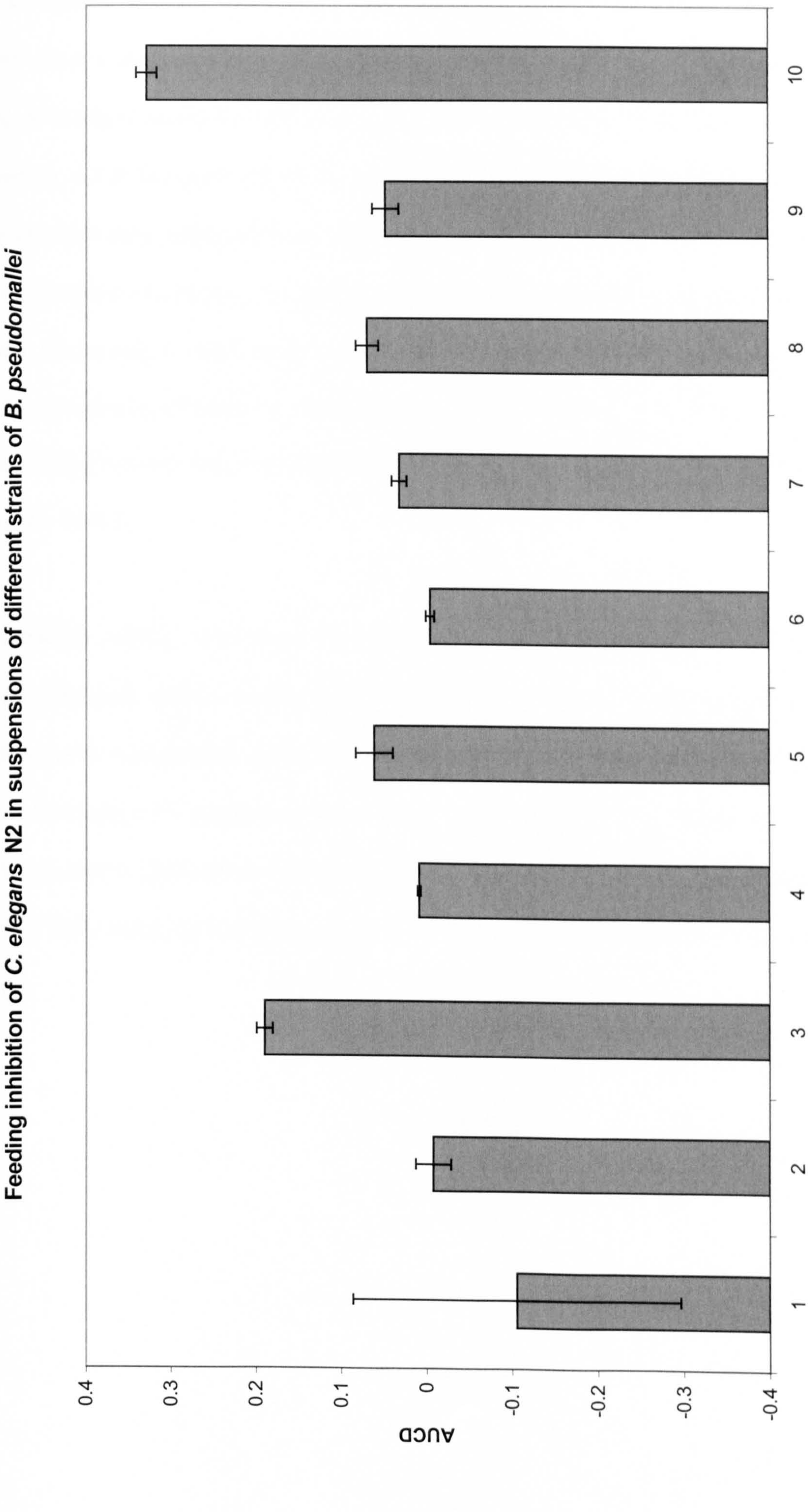


Figure: 4.13. Feeding inhibition of *C. elegans* N2 after 48 hours at 25 °C in suspensions of *B. pseudomallei* in K-medium. Strains tested were *B. pseudomallei* strain 4845 (1), strain 52 (2), strain 204 (3), strain ATCC 23343 (4), strain BRI (5), strain 42 (6), strain 2889 (7) and strain K96243 (8). Feeding rates were compared to those in suspensions of *B. pseudomallei* 576 (9) or *E. coli* OP50 (10), which were chosen to serve as controls. Error bars are the standard error of the mean AUCD at 48 hours of 8 replicates containing 50-100 L4 worms


Feeding inhibition of C. elegans N2 in suspensions of different strains of B. pseudomallei
AUCD
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
1
2
3
4
5
6
7
8
9
10
Strain tested

As a comparison to the *B. pseudomallei* strains already tested 4 *B. thailandensis* strains were also investigated. Groups of 10-20 worms were fed at 25 °C for 96 hours on lawns of each *B. thailandensis* strain and the mortality in each population monitored at 24 hour intervals. All *B. thailandensis* strains caused 100 % mortality by 96 hours. However the rate at which these strains killed *C. elegans* N2 was different depending on the strain used. *B. thailandensis* E125 gave the fastest rate of killing and caused 92 % mortality by 48 hours. Conversely *B. thailandensis* E254 gave the slowest rate of killing and only caused 55 % mortality by 48 hours. *B. thailandensis* strains E135 and E132 displayed mortality rates falling between these values giving 87 % and 77 % mortality respectively at 48 hours (Figure: 4.14.).

Calculation of TD_{50} values for *B. thailandensis* E125 confirmed that this strain was the most pathogenic of those tested with a TD_{50} value of 28 hours ± 1.6 standard error. TD_{50} values for *B. thailandensis* strains E132, E135 and E254 were 37 hours ± 1.8 standard error, 38 hours ± 2.3 standard error and 43 hours ± 3.0 standard error respectively. This indicates that *B. thailandensis* E254 was the least virulent of the strains investigated and strains E132 and E135 had intermediate levels of virulence in the plate-based mortality assay.

Figure: 4.14. Mortality of *C. elegans* N2 when fed at 25 °C for 96 hours on lawns of different wildtype *B. thailandensis* strains grown on NGM. Strains tested were *B. thailandensis* strain E125 (◆), strain E132 (■), strain E135 (▲) or strain E254 (✕). The mortality on each strain was compared to *B. pseudomallei* 576 (✶) and *E. coli* OP50 (●), which were chosen as controls. Error bars are the standard error of the mean percentage mortality of 3 replicates for each strain each containing 10-20 L4 stage worms.

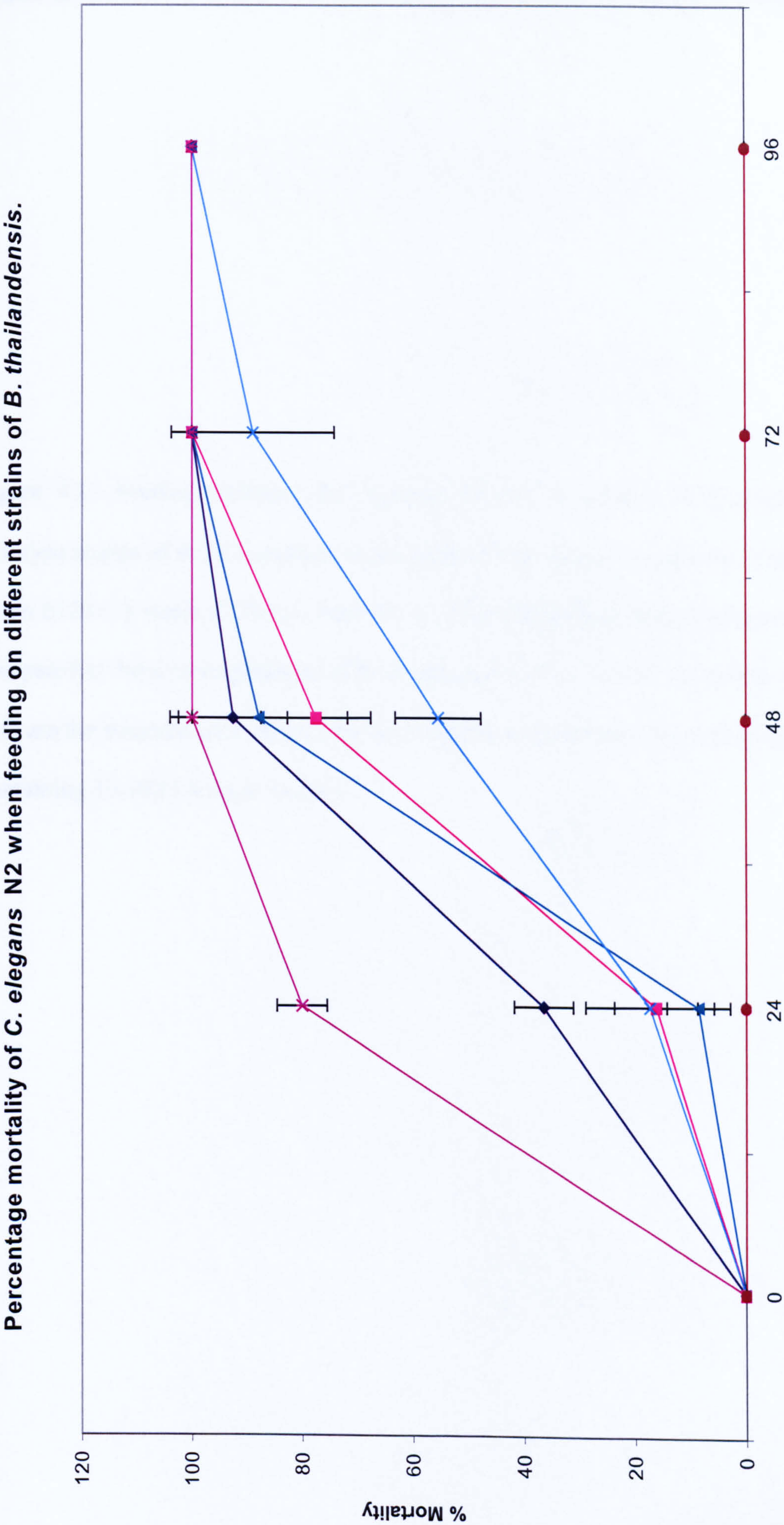
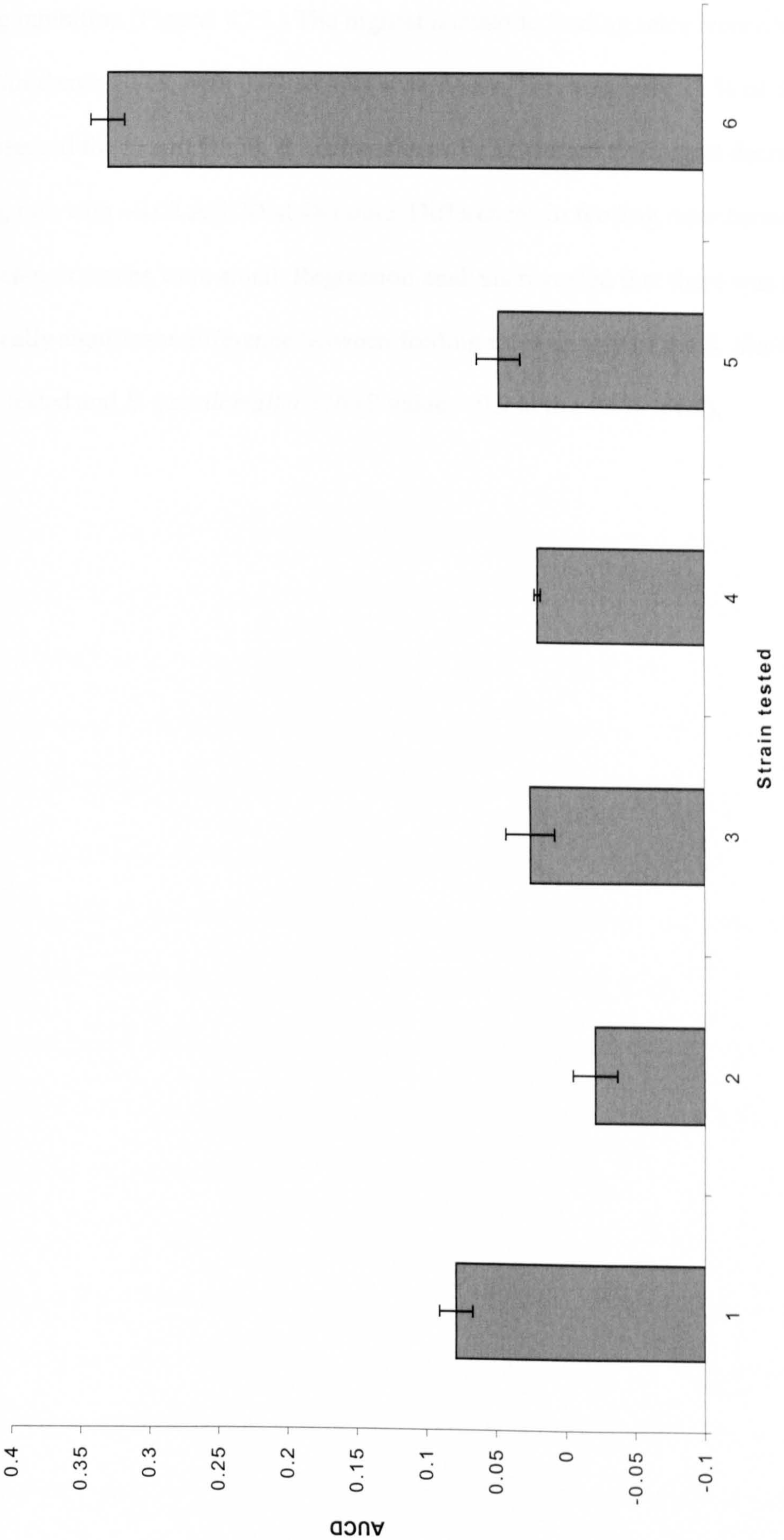

Percentage mortality of C. elegans N2 when feeding on different strains of B. thailandensis.
% Mortality
120
100
80
60
40
20
0
Time (h)
0
24
48
72
96

Figure: 4.15. Feeding inhibition of *C. elegans* N2 after 48 hours at 25 °C in suspensions of wildtype strains of *B. thailandensis* in K-medium. The strains tested were *B. thailandensis* strain E125 (1), strain E132 (2), strain E135 (3) and strain E254 (4). Feeding rates were compared to those in suspensions of *B. pseudomallei* 576 (5) or *E. coli* OP50 (6). Error bars are the standard error of the mean of 8 replicates performed for each strain, each containing 50-100 L4 stage worms.

Feeding rates of *C. elegans* N2 on different strains of *B. thailandensis*



When compared to feeding rates on *E. coli* OP50 all strains of *B. thailandensis* caused feeding inhibition (Figure: 4.15.) The highest nematode feeding rates were observed with *B. thailandensis* E125, with 0.07 AUCD at 48 hours. This was only 21 % of the feeding rate observed for *E. coli* OP50. *B. thailandensis* E132 caused the largest decrease in feeding rate with –0.02 AUCD at 48 hours. Differences in feeding rates *between B. thailandensis* strains were small. Regression analysis revealed that there was no statistically significant difference between feeding rates on any of the *B. thailandensis* strains tested and *B. pseudomallei* 576 (P value = 0.9 at the 95 % level).

4.6. Discussion.

A *C. elegans* model of bacterial infection was established using *P. aeruginosa*. The methods devised and developed were then used to establish a *C. elegans-Burkholderia* infection model. In addition to expanding the nematode model to other important bacterial species this also allowed the relevance of the model to mammalian pathogenesis to be assessed using a variety of *Burkholderia* strains.

The virulence of 20 *Burkholderia* isolates from a number of sources representing genomovars I-V of the *B. cepacia* complex, were assessed for their affect in the *C. elegans* model of infection. *B. cenocepacia sp. nov.* (formerly *B. cepacia* genomovar III) and *B. cepacia* (genomovar I) appeared to contain the most pathogenic strains in the *C. elegans* model. Those strains from *B. cenocepacia sp. nov.* which have been described as medically important such as *B. cenocepacia sp. nov.* J2315 and *B. cenocepacia sp. nov.* J415 (Govan, Brown *et al.* 1993) did cause a significant quantity of mortality in the plate-based mortality assay and also affected feeding rates in the feeding inhibition assay. However these did not induce the most severe responses in either assay. This suggests that virulence factors important in the colonisation and persistence in the CF lung that these strains posses may not be of major importance in nematode killing.

Nine strains From the *B. cepacia* complex caused ≥50% mortality of nematodes after 96 hours. From these only three were environmental isolates with the remaining six being clinical isolates. This then may indicate that clinical isolates of the *B. cepacia* complex may be better equipped for nematode killing. However those isolates that gave the slowest feeding rates in the feeding inhibition assay were environmental, suggesting a decrease in

palatability of these strains to the nematode. This result might be expected, as hypothetically the nematode would be adapted to avoid pathogenic organisms present in the same habitat. Hence strains of clinical origin may be better at killing *C. elegans* as they have adaptations that allow them to survive within animal hosts. However the origin (clinical or environmental) of the infecting strain was not related to the effect seen in either the plate-based mortality assay or the feeding inhibition assay. Thus a more complex relationship between these bacterial strains and virulence in *C. elegans* may possibly exist based on the genetic complement of virulence factors that these strains produce. Also those genomovars investigated had a small representative sample size. This may then account for the variability in pathogenic effect seen between strains and why no particular genomovar had more pathogenic representatives than another.

From the data presented, the *C. elegans* model does not provide an indication of the virulence of *B. cepacia* complex strains in mammalian hosts. A further study comparing data obtained from the screening of more strains from each genomovar in nematode and mammalian models may be required to elucidate the clinical relevance of nematodes, for studying *B. cepacia* complex pathogenesis in the mammalian host.

During observation of *C. elegans* N2 feeding on some strains from the *B. cepacia* complex a distinctive phenotype was noted. This was observed after 24 hours and was characterised by an increased side to side flexion of the body with an eventual concomitant loss in the ability to translocate across the bacterial lawn. The aetiology of this effect is presently unknown but the phenotype was reminiscent of that induced through infection of *C. elegans* with some strains of *Y. pseudotuberculosis* and *Y. pestis*. These also cause *C. elegans* to move in an aberrant manner and cause a reduction in the translocational ability of the nematode (Darby 2002). This was found to be mediated in part through the

colonisation of the nematode cuticle by the organism. (Joshua 2003). It has been suggested that this process may be interactive involving both bacterial and host recognition. This allows biofilm formation on the biotic surface of the nematode cuticle (Joshua 2003). Thus the aberrant movement phenotype seen during infection, from strains of the *B. cepacia* complex might also arise as a result of colonisation of the nematode cuticle in the form of a biofilm. Moreover this may also be an interactive process involving both bacterial and host recognition molecules as seen in *Yersinia* infection of *C. elegans*. This hypothesis is further supported by the fact that one *B. cepacia* complex strain tested also caused the formation of nose-globs.

After feeding on *B. cenocepacia sp. nov.* J415 (LMG 16654) the majority of nematodes introduced to the lawn displayed a nose-glob phenotype. All worms displaying this phenotype also moved in an aberrant manner. Nose-globs persisted until 72 hours where the phenotype was lost and worms regained normal movement indistinguishable from worms feeding on *E. coli* OP50. This suggests that the presence of nose-globs was responsible for the change in locomotive ability as normal movement was regained once nose-globs were removed. Thus, if the aberrant movement phenotype is a consequence of adherence of particles to the nematode, then it might be a behavioural mechanism designed to dislodge these from the cuticle. These would be removed by the sheer forces that would be experienced through an increase in the sinusoidal movement of the worm thus allowing the worm to regain normal movement as seen in *B. cenocepacia sp. nov.* J415 infection.

After assessment of *C. elegans srf* mutants for resistance to nose-globs no strain was found to be refractory. Nose-glob formation occurred in all mutants and wildtype nematodes with equal frequency. As these mutant *C. elegans* strains are reported to be resistant to the nose-glob phenotype caused by *Yersinia* species, the formation of nose-globs by *B. cenocepacia*

sp. nov. J415 must then occur through a different mechanism. This might involve an initial interaction with a cuticular surface antigen common in each worm strain. Alternatively the nose-globs caused by *B. cenocepacia sp. nov.* J415 may require no initial specific antigenic association and may simply be due to non-specific adherence of bacterial cells to the nematode head as the nematode moves through the bacterial lawn.

Although no *srf* mutant tested was less susceptible to nose-glob formation, *C. elegans srf*-3 was generally more susceptible to nose-glob formation (Figure: 4.7.). This strain was also shown to be more susceptible to mortality caused by *B. cenocepacia sp. nov.* J415 (Figure: 4.8.). Thus the mutation in *C. elegans srf*-3 may allow an enhanced susceptibility to *B. cenocepacia sp. nov.* J415 infection. Comparison of mortality rates when fed on *P. aeruginosa* PA14 over 96 hours revealed no difference in the mortality between either nematode strain. This suggests that the interaction observed between *C. elegans srf*-3 and *B. cenocepacia sp. nov.* J415 is a specific one that apparently results in an increase in mortality.

A *C. elegans–Burkholdeia* infection model was established through investigation of the *B. cepacia* complex. This was then was then used to investigate wildtype strains of *B. pseudomallei* and *B. thailandensis* in both the plate-based mortality assay and the feeding inhibition assay. This allowed a comparison to be made to the *Burkholderia* strains already tested and facilitated the investigation of any similar strain dependant differences in pathogenicity as observed for strains of the *B. cepacia* complex.

Worms killed by strains of *B. pseudomallei* and *B. thailandensis* were indistinguishable through microscopic examination and no specific phenotypes associated with particular strains were noted as described for strains from the *B. cepacia* complex. Killing appeared

to progress as previously reported (O' Quinn, Wiegand *et al.* 2001; Gan 2002) and 100 % of nematodes were killed by 96 hours by the majority of both species with the exception of *B. pseudomallei* 42, which killed only 41 % of nematodes by the same time-point. The reason for this difference in killing rate when compared to *B. pseudomallei* 576 is presently unknown. The possibility exists that *B. pseudomallei* strain 42 represents a naturally occurring mutant defective in toxin production. The production of a paralytic toxin has been implicated in the rapid onset of mortality in *C. elegans-B. pseudomallei* interactions (O' Quinn, Wiegand *et al.* 2001). Thus the lack of production of this toxin may produce results that are similar to those seen with the *B. cepacia* complex, for which only infection-mediated death is important. Alternatively, as *B. pseudomallei* 42 gave similar results to that of strains from the *B. cepacia* complex this strain may indeed be a strain from the *B. cepacia* complex that has been wrongly classified as a *B. pseudomallei* isolate. These species are very similar both genotypically and phenotypically and differentiation on a physical and chemical basis is difficult. This has lead to the misidentification of *B. pseudomallei* strains as *B. cepacia* complex isolates on a number of occasions (Kanai 1994). No further effort to characterise this strain has been made. However it may warrant further investigation as the lack of toxin production may facilitate dissection of *C. elegans -B. pseudomallei* interactions. Killing through toxin-mediated processes is rapid; this may hide any other effects of the infection caused by the organism. Thus a toxin-negative strain of *B. pseudomallei* would allow the reported intestinal infection of *C. elegans* to be studied over a longer period.

B. pseudomallei K96243 caused 100 % mortality at 96 hours in the plate-based mortality assay. However it was the only other strain that displayed a significantly different rate of killing at 24 hours when compared to *B. pseudomallei* 576. The calculated TD_{50} value of 35 hours ± 1.5 standard error was also higher than the other wildtype *B. pseudomallei*

strains assessed. This suggests a decrease in virulence in the *C. elegans* nematode model of infection. *B. pseudomallei* K96243 has been shown to be less virulent in mammalian models of infection than *B. pseudomallei* 576 (Dr. T. Atkins, Dstl. Pers. Comm.). This then indicates that strain-dependent differences in virulence observed in mammalian models of infection may also be mirrored in the *C. elegans–Burkholderia* infection model.

When tested in the feeding inhibition assay strain-dependant differences in virulence were also observed between *B. pseudomallei* strains. However these did not mirror those seen in the plate-based mortality assay as different strains displayed increased feeding rates and hence reduced levels of virulence in the assay. Hence the order of virulence seen in the plate-based mortality assay is not observed in the feeding inhibition assay. This suggests that strains that cause significant mortality do not always cause a reduction in feeding rate. Furthermore this also shows that different mechanisms may be responsible for mortality and feeding inhibition in *C. elegans-Burkholderia* interactions.

When investigated in the plate-based mortality assay all strains of *B. thailandensis* caused 100% mortality by 96 hours. When TD_{50} values were calculated for each strain these were found to be very similar to that of the *B. pseudomallei* strains tested. This indicates that some cross-over in virulence is apparent between *B. thailandensis* and *B. pseudomallei* in *C. elegans* as shown by the similarities in TD_{50} values. The factors responsible for nematode killing have so far been poorly characterised. Hence there may be a subset of virulence factors important in nematode pathogenicity that are possessed by some strains of both *B. pseudomallei* and *B. thailandensis* but not others. This would then account for the similarities in pathogenicity seen between these species in the nematode model of infection.

O' Quinn *et al* have showed previously that *B. thailandensis* was more pathogenic than *B. pseudomallei* in a plate-based mortality assay (O' Quinn, Wiegand *et al.* 2001). However Gan *et al* have shown *B. thailandensis* to be less pathogenic than *B. pseudomallei* in a similar assay (Gan 2002). The majority of *B. thailandensis* strains assessed in this investigation were less virulent than *B. pseudomallei* in *C. elegans*, under the plate-based mortality assay conditions used in this study. These data then support the findings of Gan *et al* and show that *B. thailandensis* is indeed less virulent in the *C. elegans* model of infection. When the same strains of *B. thailandensis* were tested in feeding inhibition assay the order of virulence was markedly different from that noted in the plate-based mortality assay. Differences in feeding rate between strains were very small and statistical analysis showed that there were no observable differences in the feeding inhibition caused by each strain.

B. thailandensis is believed to be relatively avirulent and is less virulent in animal models than *B. pseudomallei* (Smith, Angus *et al.* 1997; Brett, Deshazer *et al.* 1998; Gan 2002). Although *B. thailandensis* kills nematodes it was also found to be less virulent overall than then *B. pseudomallei* in the *C. elegans-Burkholderia* infection model. Thus these results are akin to the order of virulence displayed in vertebrates. This again shows that *C. elegans* may be a suitable model for the study of *B. pseud*omallei pathogenesis in the mammalian host.

The discrepancies observed between the plate-based mortality assay and the feeding inhibition assay may be due to the differences in media used in both assays as *C. elegans*-pathogen interactions have been shown to be heavily media dependant (Darby, Cosma *et al.* 1999; Tan, Miklos *et al.* 1999; Gan 2002; Tan 2002). In the plate-based mortality assay bacteria are grown on a low osmolarity nutrient-agar (NGM), which allows normal

bacterial growth. Conversely the feeding inhibition assay utilises K-medium which is an isotonic solution that contains no nutrients for bacterial growth. Hence bacterial cells may become stressed and possibly respond differently to their nutritionally replete counterparts grown on NGM. Additionally the difference in infectious dose received may cause the differences observed. In the feeding inhibition assay worms are introduced to a relatively dilute bacterial suspension and allowed to feed (starting OD of approximately 1.2). However in the plate-based mortality assay worms are introduced to a bacterial lawn and crawl through it as they feed. Thus the worms would be exposed to and ingest many more bacterial cells at any one time. This then may also account for the difference in response seen in both assays.

Screening a large panel of *Burkholderia* genus isolates has shown that *C. elegans* N2 is susceptible to a variety of *B. cepacia* complex, *B. pseudomallei* and *B. thailandensis* strains. A number of strain-dependant differences in pathogenicity in the nematode model have also been noted and are particularly prevalent between the *B. cepacia* complex isolates tested. These differences are also seen in *B. pseudomallei* and *B. thailandensis* strains but to a lesser extent. Importantly for this study, the differences in virulence seen in the plate-based mortality assay within *B. pseudomallei* strains compared to strains of *B. thailandensis* mimics that seen in conventional animal models of infection. This shows that *C. elegans* is an applicable and relevant model of *B. pseudomallei* infection and may be used to study further the basis of *B. pseudomallei* pathogenesis in the mammalian host.

Chapter: 5. Study of *B. pseudomallei* infection of the *C. elegans* gut.

5.1. Introduction.

One of the most basic methods for the study of host-pathogen interactions is the direct observation of the pathogenic process *in situ*. This can be achieved through the use of microscopic techniques including electron microscopy. Such methods allow the study of the discrete binding events that lead to colonisation of a host and provide valuable information concerning the early events of the infection such as the cellular localisation of bacterial cells within infected host tissues. It also permits the examination of the histopathology induced by the action of virulence factors produced by bacterial pathogens, which is often the route cause of disease.

5.2. Investigation of host-pathogen interaction in *C. elegans* using TEM.

To compliment the other methods that will be used to study *B. pseudomallei* pathogenesis in *C. elegans*, the interaction between host and pathogen at the cellular level was assessed using TEM. Approximately 1000-2000 *C. elegans* N2 worms were allowed to feed on either *B. pseudomallei* 576 or *E. coli* OP50 for a total of 24 hours at 25 °C. The samples were then fixed overnight *in situ* through the addition of 5 % (v/v) glutaraldehyde. Fixed samples were washed to remove residual glutaraldehyde before final processing and visualisation by thin section TEM (Section: 2.2.2.).

After 24 hours of feeding in suspension, thin section TEM revealed that both intact *E. coli* OP50 and *B. pseudomallei* 576 cells were present within the intestinal lumen of the nematode

(Figure: 5.1 and 5.2.). No bacterial invasion of tissues surrounding the intestine by *B. pseudomalle*i 576 cells were seen in any sample investigated. There was also no observable tissue damage caused by the pathogen when compared to feeding on *E. coli* OP50. However a large amount of what may be bacterial debris was observed in the interstitial spaces surrounding *B. pseudomallei* 576 cells. Amongst this darkly staining material a number of what appeared to be membrane bound vesicular structures of approximately 100-200 nm in diameter were also observed (Figure: 5.2.). These vesicles were not evident when nematodes were allowed to feed on *E. coli* OP50 (Figure: 5.1.).

Figure: 5.1. Electron-micrograph showing a transverse cross-section through the lumen of the *C. elegans* N2 intestine. Approximately 1000-2000 L4 stage nematodes were fed on suspensions of *E. coli* strain OP50 with an approximate OD_{550} nm of 1.2 for 24 hours at 25 °C. Samples were then fixed in situ through the addition of 3 volumes of 5 % glutaraldehyde (v/v) before processing for thin section TEM. Intact *E. coli* (B); gut mucosa (GM); gland cell duct (GD); motor neurones (MN). Section thickness 70 nm. Scale bar represents 2 μm.

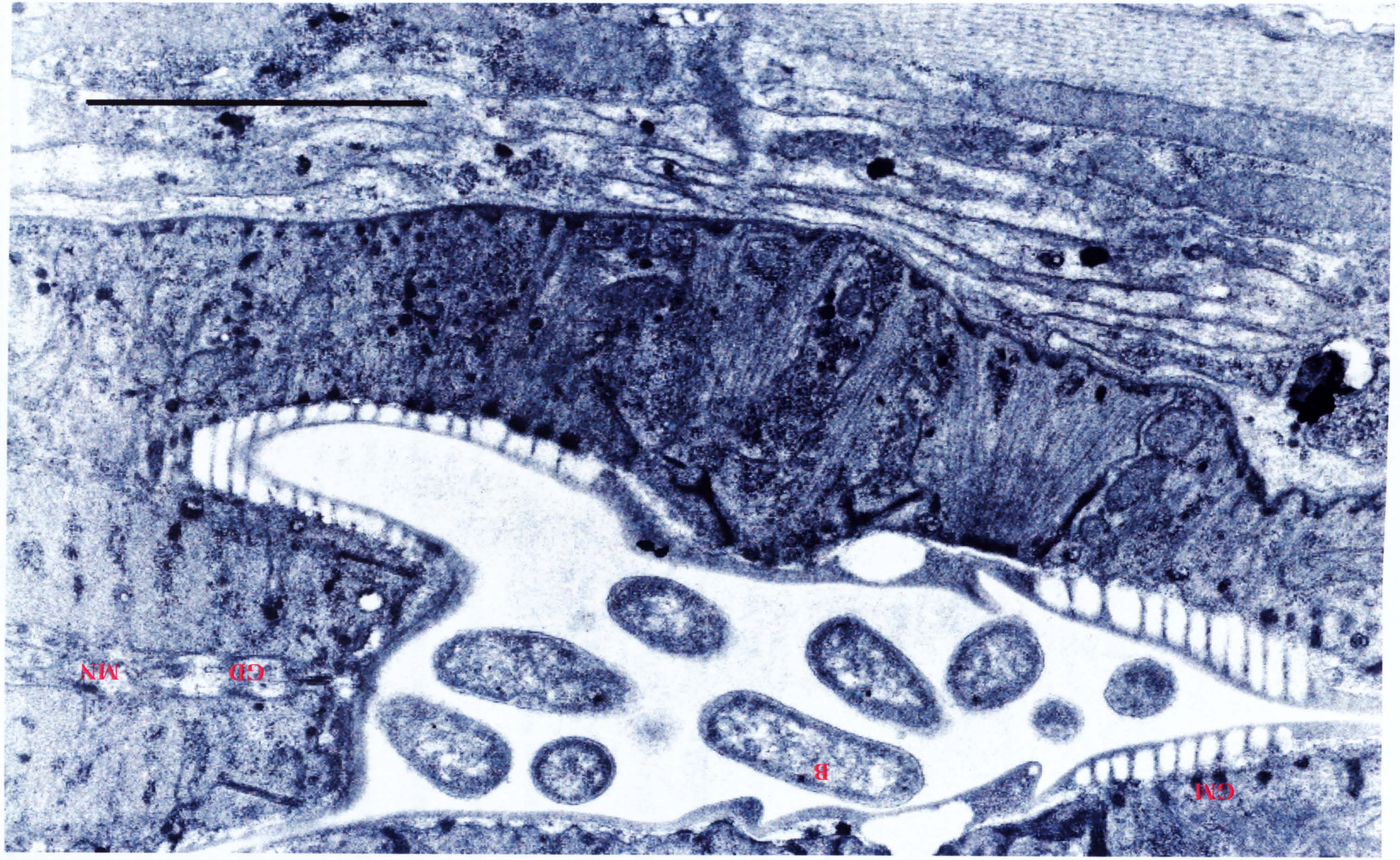

MN
GD
B
CM

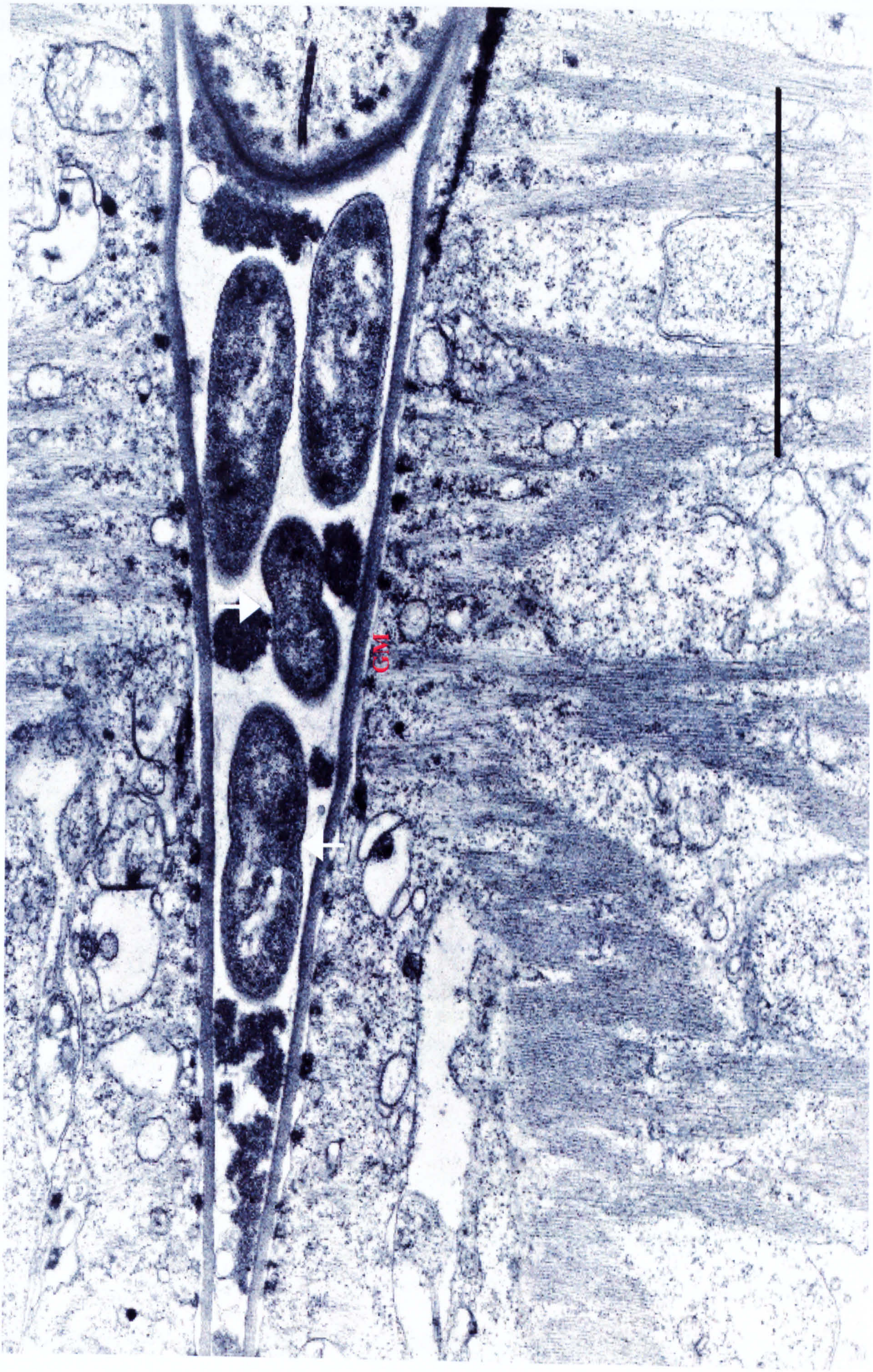
Figure: 5.2. Electron-micrograph showing a transverse cross-section through the lumen of the *C. elegans* N2 intestine. Approximately 1000-2000 L4 stage nematodes were fed on suspensions of *B. pseudomallei* 576 with an approximate OD_{550} nm of 1.2 for 24 hours at 25 °C. Samples were then fixed in situ through the addition of 3 volumes of 5 % glutaraldehyde (v/v) before processing for thin section TEM. Intact *B. pseudomallei* cells (B); bacterial debris (D); gut mucosa (GM); gland cell duct (GD); motor neurones (MN). White arrowheads indicate unidentified membrane bound vesicular structures. Section thickness 70 nm. Scale bar represents 4 μm.


MN
GD
P
B
GM

Figure: 5.3. Electron-micrograph showing a transverse cross-section through the lumen of the *C. elegans* N2 intestine. Approximately 1000-2000 nematodes were fed on suspensions of *B. pseudomallei* 576 with an approximate OD_{550} nm of 1.2 for 24 hours at 25 °C. Samples were then fixed in situ through the addition of 3 volumes of 5 % glutaraldehyde (v/v) before processing for thin section TEM. Intact *B. pseudomallei* cells (B); gut mucosa (GM); gland cell duct (GD); motor neurones (MN). Grey arrowhead indicates an unidentified double membrane bound vesicular structure and large black arrowhead indicates a dividing *B. pseudomallei* cell. Section thickness 70 nm. Scale bar represents 2.5 μm.


B
GD
GM
MN

Figure: 5.4. Thin Layer electron-micrograph showing division of *B. pseudomallei* 576 in the intestinal lumen of a *C. elegans* L4 stage nematode. Approximately 1000-2000 L4 stage nematodes were fed on suspensions of *B. pseudomallei* 576 with an approximate OD_{550} nm of 1.2 for 24 hours at 25 °C. Samples were then fixed in situ through the addition of 3 volumes of 5 % glutaraldehyde (v/v) before processing for thin section TEM. Intact *B. pseudomallei* (B); gut mucosa (GM); musculature (M). White arrows indicate dividing *B. pseudomallei*. Section thickness is 70 nm. Scale bar represents 2 μm.


GM

On further examination of thin sections some *B. pseudomallei* 576 cells were seen to be in the process of cellular division (Figure: 5.3. and 5.4.). In contrast this was not observed for those samples where worms had only fed on *E. coli* OP50. These samples contained whole cells but no evidence of bacterial replication as exemplified by figure: 5.1.

5.3. Discussion.

The nematode *C. elegans* has been suggested to be a good model of *B. pseudomallei* pathogenesis in mammals. Use of the model has allowed the characterisation of novel virulence factors at the molecular level that are also apparently important in mammalian pathogenesis (O' Quinn, Wiegand *et al.*2001; Gan 2002). However these studies have not investigated the host-pathogen interactions occurring at the microscopic level that may contribute to pathogenesis in both nematodes and mammalian species. This was addressed in this investigation through the use of TEM.

After 24 hours of feeding in suspensions of both *B. pseudomallei* 576 and *E. coli* OP50 whole bacterial cells were observed within the gut lumen (Figures: 5.1. and 5.2.). This suggests that disruption of bacterial cells by the grinder within the pharynx is incomplete regardless of the pathogenic potential of the organism, and that viable bacterial cells enter the gut.

In sections of worms which had been feeding on *B. pseudomallei* 576 (Figures: 5.3. and 5.4.), a number of bacterial cells were identified which appeared to be in the process of cellular division. No dividing bacterial cells were observed when worms had fed on *E. coli* OP50 (Figure: 5.1.). Thus although whole *E. coli* OP50 cells pass the grinder and enter the gut no replication was observed. The ability to survive and replicate within the nematode intestine may then represent a fundamental difference between *B. pseudomallei* and *E. coli* that would in part account for the differences in virulence observed. After 24 hours feeding on *B. pseudomallei* 576 no bacterial cells were observed within cells immediately surrounding the nematode gut. Also, no multi-nucleate giant cells were observed which are a characteristic of

B. pseudomallei infection in mammalian cell culture (Harley 1998). Additionally no cells were seen adhering to the surface of the gut mucosa (Figure: 5.2.). This suggests that cells lining the gut may be refractory to *B. pseudomallei* 576 infection. The mucosa of the *C. elegans* gut appears as a thick darkly staining layer (Figure: 5.2.) in TEM thin sections, which may represent an impermeable barrier to the invasion efforts of the organism. The refractory nature of the cells lining the *C. elegans* intestine to bacterial infection would be expected. As *C. elegans* has only a rudimentary non-specific immune system (Section: 1.5.4.5.) The ability to deny potentially detrimental intracellular pathogens access into underlying tissues could then be a requirement for nematode survival. This is supported by recent studies by Herndon *et al* (Herndon 2003) who showed that the intestine is impermeable to many bacterial pathogens and invasion of underlying tissues rarely occurs until after nematode death. Furthermore, although no intracellular bacterial cells were noted in thin sections, this may imply that none were present in the samples analysed.

When studying the relationship of a bactivourous nematode with its food source the obvious place to examine for any pathogenic interaction would be in the intestine. However this assumption may be misplaced and invasion of other areas of the body may occur. It has been shown recently that some pathogens can invade the vulva and uterus of infected worms and may even invade the sensory organs of the head and pharyngeal muscles (Herndon 2003). Thus intracellular bacterial cells may not have been evident in these samples simply due to the localisation of the sections through the intestinal lumen. Inspection of thin-sections through other areas of the nematode body was not possible in this study due to time constraints. However a further study screening thin-sections taken through other areas of the nematode body, such as the head and vulva may reveal intracellular or invading bacterial cells missed in the original screen.

An interesting observation made during this study was the presence of what appeared to be membrane-bound vesicular structures surrounding bacterial cells within the *C. elegans* intestinal lumen (Figure: 5.3.). This phenomenon was only observed in those samples where nematodes were allowed to feed on *B. pseudomallei* 576 and not *E. coli* OP50. The origin of these structures is elusive but they may be bacterially derived. The cell wall in Gram-negative bacteria is a dynamic structure that has features not seen in Gram-positive species. During bacterial growth vesicles are constantly discharged from the outer-membrane. These are 50 to 250 nm-diameter structures that are spherical, bilayered membranous structures. They posses outer-membrane proteins, LPS, phospholipids and periplasmic constituents that are situated as they would normally be found in the parent bacterium (Beveridge 1999). Membrane vesicles are found emanating from Gram-negative bacteria growing on solid media, in liquid media, in swarming cultures and also in natural environments.

The vesicular structures observed in thin sections of worms fed on *B. pseudomallei* 576 appear to be spherical, posses a double membrane and range between 100 and 200 nm in diameter (Figure: 5.3.). Thus these conform to the general observations made of membrane vesicles from other Gram-negative bacterial species. As the production of membrane vesicles is only observed in actively growing bacterial cultures (Beveridge 1999) this further indicates that the *B. pseudomallei* 576 cells present in electron-micrographs were viable at the point of fixation. Conversely as no vesicle production was noted in samples containing *E. coli* OP50 this also further demonstrates that these cells may have been non-viable.

In some species such as *P. aeruginosa*, membrane vesicles have been shown to function in the killing of other Gram-negative and Gram-positive bacterial species, through the delivery of degradative hydrolytic enzymes to the cell surface of target organisms (Li 1998). However it

has also been suggested that they may function in pathogenicity in the mammalian host through delivery of virulence factors (Beveridge 1999). For example, the membrane vesicles of *P. aeruginosa*, *P. mirabilis* and *S. marcescens* can package phospholipase C, proteases, and hemolysins. These are protected from the host serum constituents that act to inactivate them by the lipid bilayer of the vesicle. They can then be delivered to their site of action in an undiluted form where they cause a variety of histopathological changes (Beveridge 1999).

Death of *C. elegans* through *B. pseudomallei* infection is reported to be mediated in part through the action of a diffusible toxin that may effect L-type voltage-gated Ca^{2+} channels resulting in eventual muscular paralysis and death (O' Quinn, Wiegand *et al.* 2001). This has been shown to be an active process and require the presence of live bacteria for a maximal killing effect. As membrane vesicle production only occurs in actively growing bacteria the toxin may then be delivered within these structures and cause the eventual paralysis observed. Additionally *B. pseudomallei* produces a wide variety of degradative enzymes (Ellis 1999). However the secretion of digestive exoenzymes through the general secretion machinery has been shown to not be important in killing (O' Quinn, Wiegand *et al.* 2001; Kothe 2003). Thus other methods of secretion may be involved in the delivery of these enzymes to affected tissues including membrane vesicles. Techniques such as immune electron microscopy, using antibodies raised against the *B. pseudomallei* 576 outer membrane might allow the origin of these structures to be determined and help to provide information on their role in *B. pseudomallei* pathogenesis in nematodes.

Taken together these observations suggest that *B. pseudomallei* 576 infection of *C. elegans* does not mimic that seen in the mammalian host. Bacteria entering the intestine appear viable but no invasion of intestinal cells is observed. Also no change in nematode tissues surrounding

the gut is observable after 24 hours of exposure to the organism. Death may then occur through the co-ordinated action of toxic factors that act to paralyse and eventually degrade tissues liberating nutrients for bacterial growth, which possibly involves the production of membrane vesicles. Further studies would be needed to assess the importance of these observations to *B. pseudomallei* pathogenesis in the nematode *C. elegans*.

Chapter: 6. Screening for attenuated *B. pseudomallei* mutants using *C. elegans*.

6.1. Introduction.

Relatively little is known concerning the molecular basis of virulence of *B. pseudomallei* in the mammalian host. A number of potential virulence factors have been identified including capsular polysaccharide, pili, toxins and degradative enzymes (Ellis 1999). However many of these remain poorly characterised and their role in the pathogenicity of the organism is unclear.

The *C. elegans-Burkholderia* infection model was established in this study using a variety of *Burkholderia* strains. The results suggest that *C. elegans* is a relevant model for the study of *B. pseudomallei* virulence in the mammalian host. This finding allowed the nematode model to be used for the investigation of the molecular basis of *B. pseudomallei* virulence. Initially this involved the screening of *B. pseudomallei* mutants that have already been shown to be attenuated in mammalian models of infection, in the *C. elegans-Burkholderia* model. This would show if the *C. elegans-Burkholderia* model was capable of detecting mutations that lead to a decrease in virulence in both nematodes and mammalian models, thus validating the screening method to be used.

Following validation the nematode model was used to screen a pool of transposon mutants already available at Porton for those that displayed an attenuated phenotype. Any mutants isolated in *C. elegans* may also show a reduced virulence in mammalian models of infection. Thus, characterisation of mutations responsible for this phenotype, might provide additional and important information regarding the molecular basis of *B. pseudomallei* pathogenesis in the mammalian host.

6.2. Screening of *B. pseudomallei* mutants for a reduction of virulence in a nematode model of infection.

In Chapter 4, the *C. elegans-Burkholderia* infection model was shown to be able to detect differences in virulence between wildtype strains of *B. pseudomallei*. These differences were also shown to mirror the differences in virulence of these strains in mammalian models of infection. In this chapter, the ability of the *C. elegans-Burkholderia* infection model to detect bacterial mutants with reduced virulence in nematodes was assessed.

6.2.1. Screening of an attenuated *B. pseudomallei* K96243 mutant in *C. elegans* N2.

A *B. pseudomallei* mutant strain (Δ*pilA*) was obtained that had been previously shown to have a reduced virulence in mammals when compared to its parent strain *B. pseudomallei* K96243. This mutant carries a mutation in the pilin structural subunit of the type IV pili. These structures have a role in DNA uptake, protein secretion, and adherence. They are also involved in a form of movement called twitching motility (Christie 2000).

The mutant Δ*pilA* was tested for its virulence towards nematodes in the plate-based mortality assay. Groups of 10-20 L4 stage *C. elegans* N2 worms were fed for 96 hours at 25 °C on lawns of *B. pseudomallei* Δ*pilA* grown on NGM and the mortality in the population recorded daily. Both *B. pseudomallei* K96243 and *B. pseudomallei* Δ*pilA* caused 100 % mortality of nematodes by 72 hours post-exposure (Figure: 6.1.). However the mutant strain Δ*pilA* killed *C. elegans* N2 at a slower rate than that of *B. pseudomallei* K96243 (Figure: 6.1.).

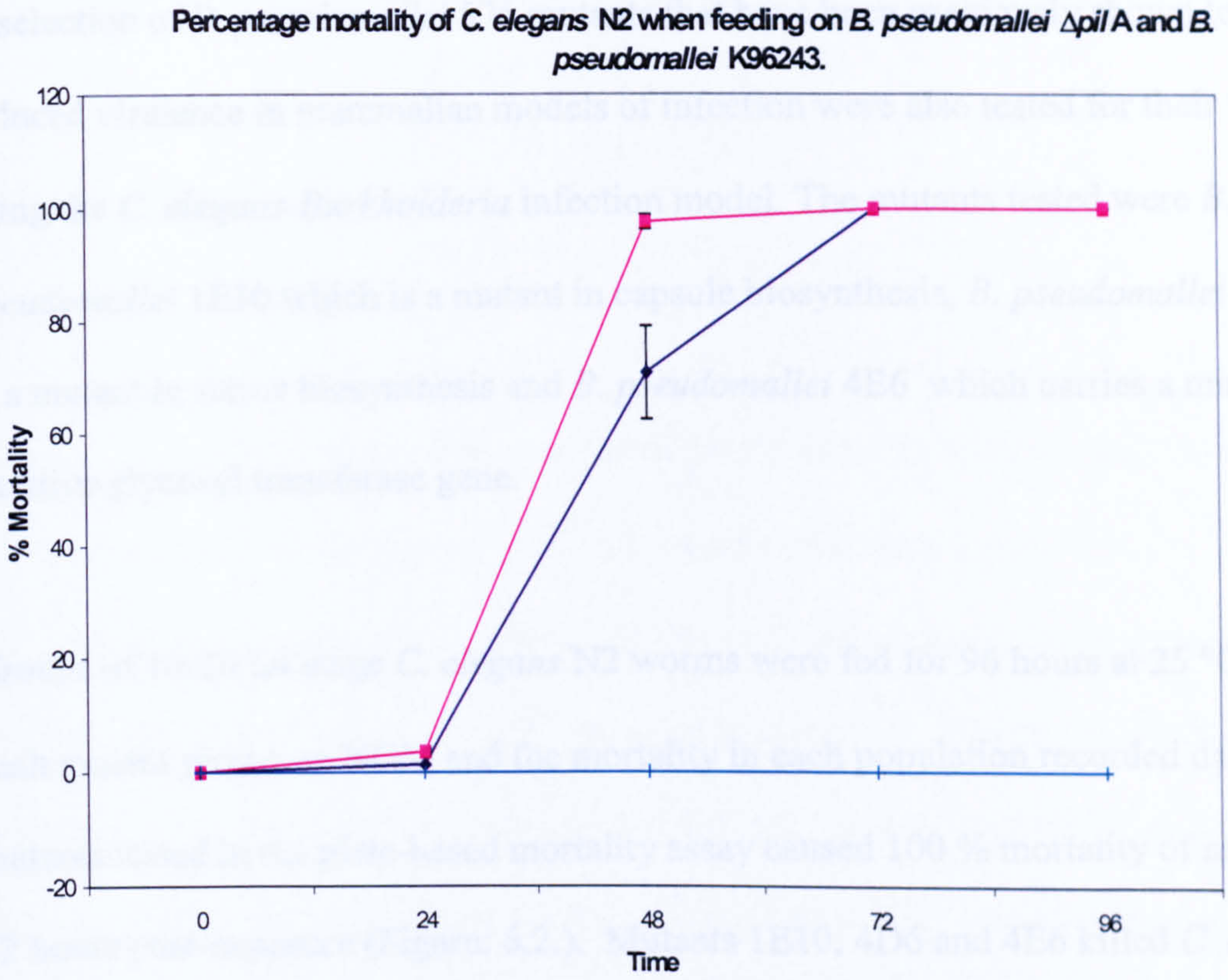


Figure: 6.1. Assessment of differences in virulence between *B. pseudomallei* K96243 (■) and the mutant strain *B. pseudomallei* Δ*pilA* (◆). Groups of 10-20 L4 stage worms were fed at 25 °C for 96 hours on lawns of either mutant or wildtype strain and the mortality recorded at 24 hour intervals. Feeding on *E. coli* OP50 (▮) was used as a control. Error bars are the standard error of the mean of three replicates performed for each strain tested.

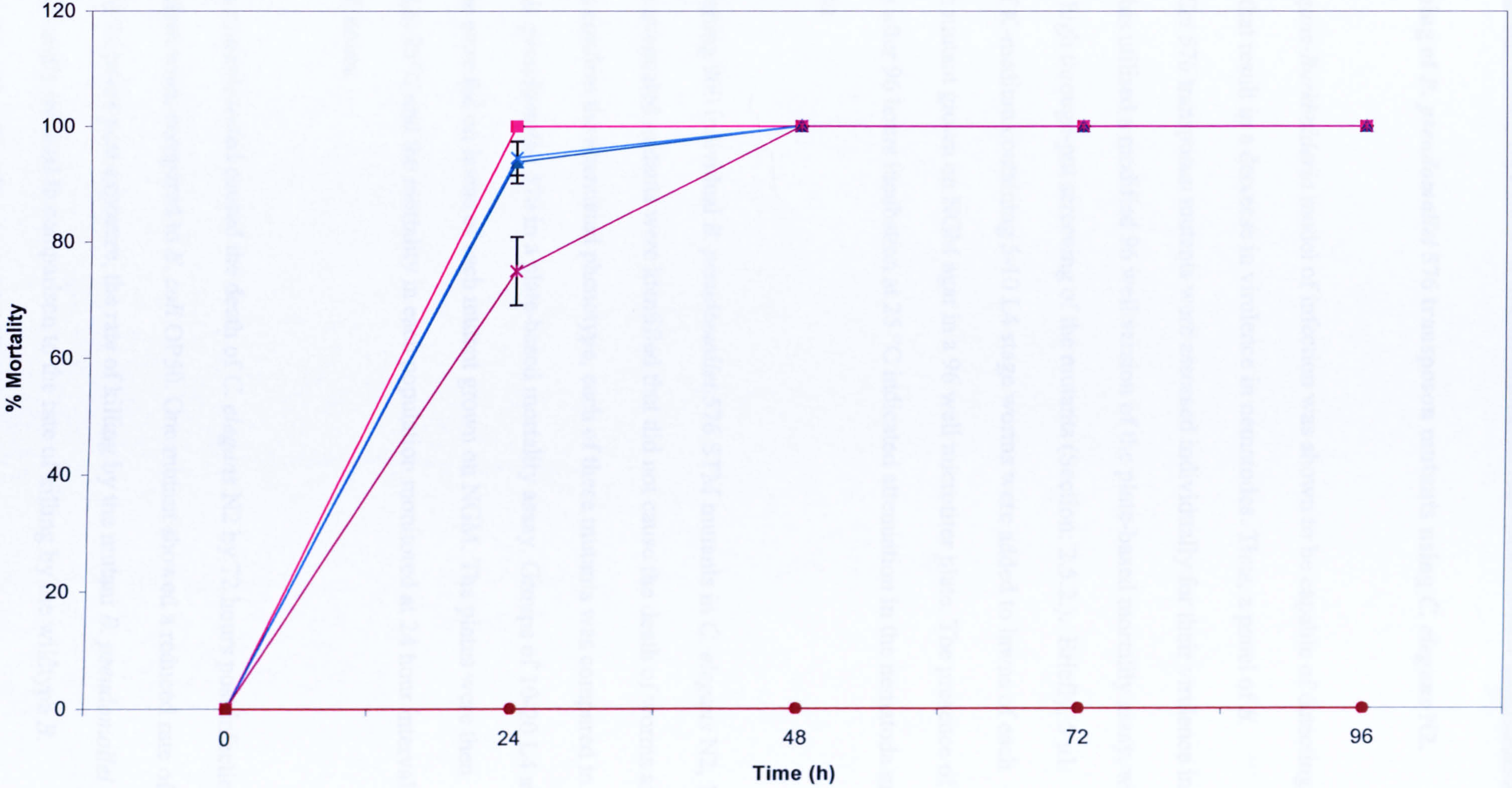
6.2.2. Screening of attenuated *B. pseudomallei* 576 mutants in *C. elegans* N2.

A selection of *B. pseudomallei* 576 mutants that have been previously shown to have a reduced virulence in mammalian models of infection were also tested for their virulence using the *C. elegans-Burkholderia* infection model. The mutants tested were *B. pseudomallei* 1E10 which is a mutant in capsule biosynthesis, *B. pseudomallei* 4D6, which is a mutant in serine biosynthesis and *B. pseudomallei* 4E6 which carries a mutation in a putative glycosyl transferase gene.

Groups of 10-20 L4 stage *C. elegans* N2 worms were fed for 96 hours at 25 °C on lawns of each mutant grown on NGM and the mortality in each population recorded daily. All mutants tested in the plate-based mortality assay caused 100 % mortality of nematodes by 72 hours post-exposure (Figure: 6.2.). Mutants 1E10, 4D6 and 4E6 killed *C. elegans* more quickly than *B. pseudomallei* strain 576.

Figure: 6.2. Assessment of differences in virulence between wildtype and mutant strains of *B. pseudomallei* 576. Groups of 10-20 L4 stage worms were fed at 25 °C for 96 hours on lawns of either *B. pseudomallei* strain 576 (✱) or *B. pseudomallei* mutant strains previously shown to have a reduced virulence in mammals. The mutant strains tested were *B. pseudomallei* 1E10 (■), *B. pseudomallei* 4D6 (▲) or *B. pseudomallei* 4E6 (×). Feeding on *E. coli* OP50 (●) was used as a control. Error bars are the standard error of the mean of three replicates performed for each strain tested.

Percentage mortality of *C. elegans* N2 when fed on mutant strains of *B. pseudomallei.*



6.3. Screening of *B. pseudomallei* 576 transposon mutants using *C. elegans* N2.

The *C. elegans-Burkholderia* model of infection was shown to be capable of detecting mutations that result in a decrease in virulence in nematodes. Thus, a panel of *B. pseudomallei* 576 transposon mutants were assessed individually for their virulence in *C. elegans*. This utilised a modified 96 well version of the plate-based mortality assay, which facilitated high through-put screening of the mutants (Section: 2.5.2.). Briefly, 5 µl aliquots of K-medium containing 5-10 L4 stage worms were added to lawns of each transposon mutant grown on NGM agar in a 96 well microtiter plate. The presence of live nematodes after 96 hours incubation at 25 °C indicated attenuation in the nematode model of infection.

After screening 960 individual *B. pseudomallei* 576 STM mutants in *C. elegans* N2, 14 putatively attenuated mutants were identified that did not cause the death of worms after 96 hours. To confirm the attenuated phenotype, each of these mutants was compared to the wildtype *B. pseudomallei* 576 in a plate-based mortality assay. Groups of 10-20 L4 stage nematodes were fed on lawns of each mutant grown on NGM. The plates were then incubated at 25°C and the mortality in each population monitored at 24 hour intervals for a total of 96 hours.

All of the mutants tested caused the death of *C. elegans* N2 by 72 hours post infection and were virulent when compared to *E. coli* OP50. One mutant showed a reduced rate of killing. At 24 hours post exposure, the rate of killing by the mutant *B. pseudomallei* 6H2 was significantly reduced in comparison to the rate of killing by the wildtype *B. pseudomallei* 576, with a P value of 0.037. The mutant *B. pseudomallei* 6H2 was selected

for further study through a comparison to *B. pseudomallei* 576 in the plate-based mortality assay and the feeding inhibition assay.

Groups of 10-20 L4 stage *C. elegans* N2 nematodes were fed at 25 °C, for a total of 92 hours, on lawns of *B. pseudomallei* 576 or *B. pseudomallei* 6H2 grown on NGM. Both *B. pseudomallei* 6H2 and *B. pseudomallei* 576 caused 100% mortality by 92 hours. At 32 hours post-exposure a difference in the rate of killing was observed between the strains (Figure: 6.3.). However the difference was not significantly different when compared using a students t-test (P value = 0.131) or regression analysis (P value = 0.119).

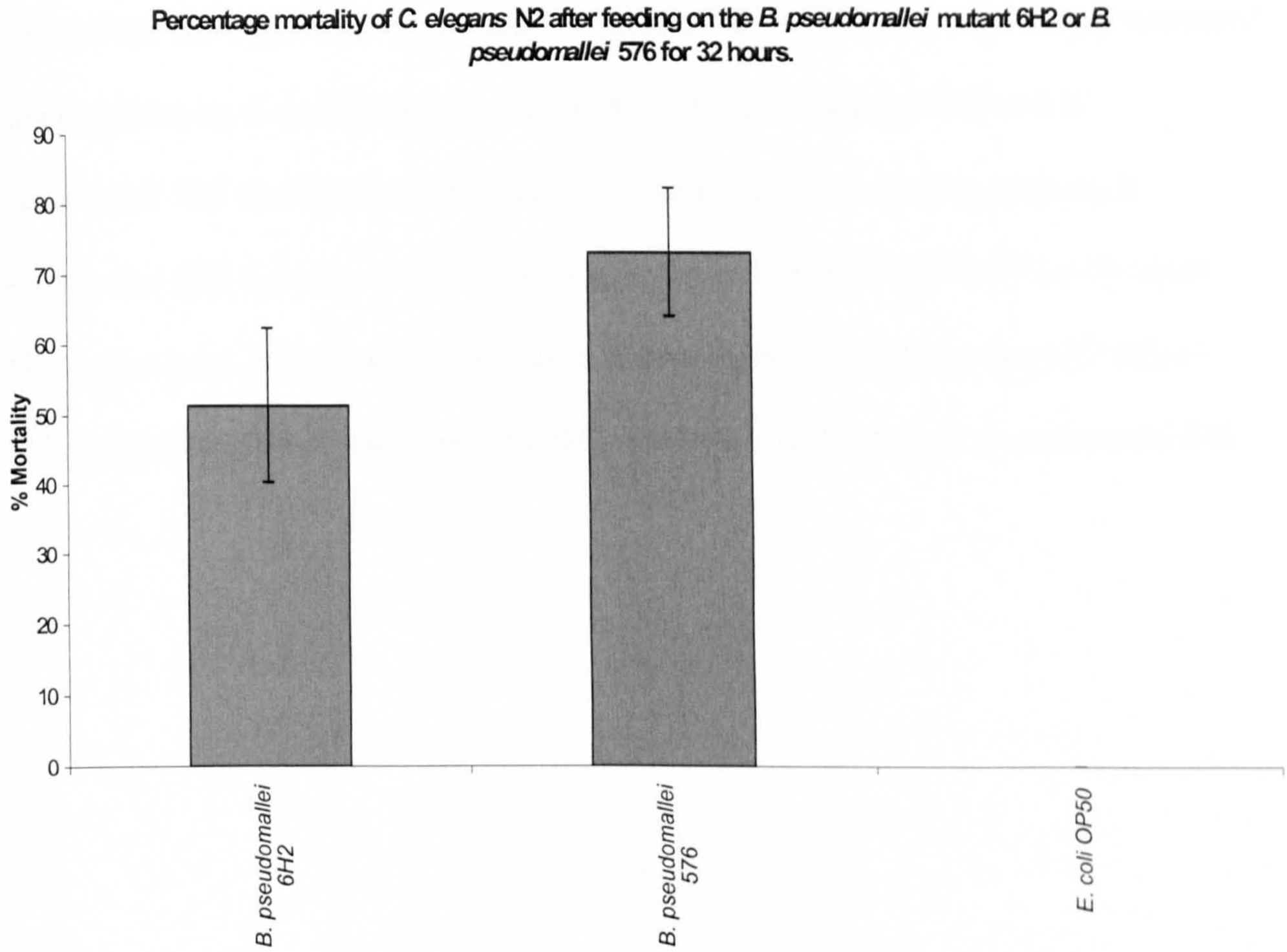


Figure: 6.3. Mortality of *C. elegans* N2 at 25 °C while feeding on *B. pseudomallei* 6H2 or *B. pseudomallei* 576 grown on NGM at 32 hours. Feeding on lawns of *E. coli* OP50 was used as a control. Error bars are the standard error of the mean percentage mortality of 3 replicates performed for each strain containing 10-20 L4 stage worms.

The virulence of *B. pseudomallei* 6H2 and the wildtype *B. pseudomallei* 576 was compared in the feeding inhibition assay. Aliquots of 50-100 L4 stage nematodes were fed in 1 ml suspensions of each strain at an approximate starting OD_{550nm} of 1.2 for 48 hours at 25 °C and the OD_{550nm} of each suspension measured at 24 hour intervals. When compared to feeding rates on *E. coli* OP50, feeding on both *B. pseudomallei* 6H2 and *B. pseudomallei* 576 was inhibited (Figure: 6.4.). However when feeding rates on *B. pseudomallei* 6H2 were compared to feeding rates in *B. pseudomallei* 576 at 48 hours, feeding rates on *B. pseudomallei* 6H2 were shown to be significantly faster (P value = 0.026) suggesting that *B. pseudomallei* 6H2 was less virulent than *B. pseudomallei* 576.

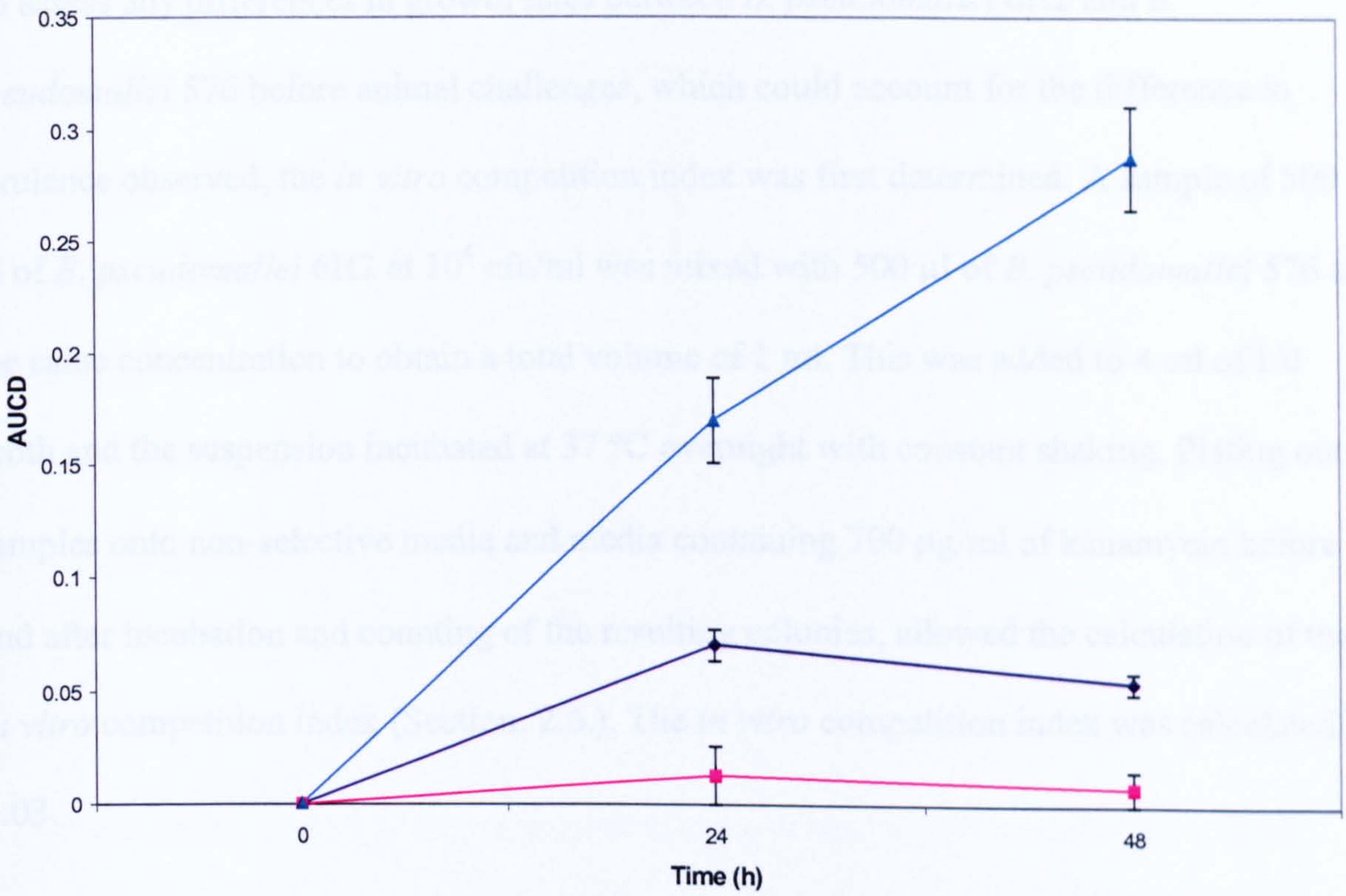


Figure: 6.4. Feeding inhibition of *C. elegans* N2 in suspensions of *B. pseudomallei* 6H2 (◆) or *B. pseudomallei* 576 (■) in K-medium at 25 °C. Feeding in *E. coli* OP50 was used as a control (▲). Error bars are the standard error of the mean AUCD of 8 replicates for each strain each containing 50-100 L4 stage worms.

6.4. Virulence of transposon mutant *B. pseudomallei* 6H2 or *B. pseudomallei* 576 in BALB/c mice.

To assess any differences in growth rates between *B. pseudomallei* 6H2 and *B. pseudomallei* 576 before animal challenges, which could account for the difference in virulence observed, the *in vitro* competition index was first determined. A sample of 500 µl of *B. pseudomallei* 6H2 at 10^4 cfu/ml was mixed with 500 µl of *B. pseudomallei* 576 at the same concentration to obtain a total volume of 1 ml. This was added to 4 ml of LB broth and the suspension incubated at 37 °C overnight with constant shaking. Plating out of samples onto non-selective media and media containing 700 µg/ml of kanamycin before and after incubation and counting of the resulting colonies, allowed the calculation of the *in vitro* competition index (Section: 2.6.). The *in vitro* competition index was calculated as 1.03.

To determine whether *B. pseudomallei* 6H2 was attenuated in a mammalian model of disease, the virulence of this strain was compared to the virulence of *B. pseudomallei* 576 in BALB/c mice. Two groups of five 6-8 week old female BALB/c mice were challenged intra-peritoneally with 10^3 cfu or 10^4 cfu of *B. pseudomallei* 6H2 or *B. pseudomallei* 576. This equated to 10 MLD or 100 MLD respectively of *B. pseudomallei* 576. The survival in each group was then monitored for a total of 21 days post-challenge (Table: 6.1.).

Challenge strain	Challenge dose (cfu)	Survivors at day 21	Mean time to death (days ± SE)
B. pseudomallei 576	10^3	3	5 ± 3.2
B. pseudomallei 6H2	10^3	2	15.5 ± 1.9
B. pseudomallei 576	10^4	0	6 ± 0.8
B. pseudomallei 6H2	10^4	2	8.6 ± 2.0

Table: 6.1. Survival of BALB/c mice after intraperitoneal injection with the mutant *B. pseudomallei* 6H2 or *B. pseudomallei* 576. Four groups of five 6-8 week old female BALB/c mice were inoculated with doses of either 10^3 cfu or 10^4 cfu *of B. pseudomallei* 6H2 or 10^3 cfu and 10^4 cfu of *B. pseudomallei* 576. Mice were observed for a total of 21 days and the survivors in each group recorded at 24 hour intervals.

After 4 days post challenge only 3 mice that had received 10^3 cfu of *B. pseudomallei* 576 remained alive. No further deaths were recorded in this group for the remainder of the experiment. No mice which had received 10^3 cfu of *B. pseudomallei* 6H2 died until day 14 when 2 mice died. At day 15 one further mouse in this group died leaving 2 survivors until the end of the experiment at day 21 (Table: 6.1.). The mean time to death for mice receiving 10^3 cfu of *B. pseudomallei* 6H2 and *B. pseudomallei* 576 was 15.5 days and 5 days respectively.

After 5 days post-challenge 3 mice had died in the group receiving 10^4 cfu of *B. pseudomallei* 576. The remaining 2 mice in this group died by day 7 resulting in a mean time to death of 6 days. At day 5 in the group receiving 10^4 cfu of *B. pseudomallei* 6H2 2 mice died. A further 1 mouse died at day 15 and no further deaths were recorded. (Table: 6.1.). The mean time to death of mice given 10^4 cfu of *B. pseudomallei* 6H2 was 8.6 days.

As a further comparison to elucidate any differences in colonisation ability between the wildtype *B. pseudomallei* 576 and the mutant *B. pseudomallei* 6H2 *in vivo* the competitive index was also calculated in BALB/c mice (Section: 2.5.). Five mice were inoculated through intra-peritoneal injection with 10^2 cfu of mutant and 10^2 cfu wildtype bacteria (total 10^4 cfu). After 48 hours the spleens of each animal were removed, homogenised and dilutions plated onto selective media and non-selective media as already described. These were incubated at 37 °C overnight and the colonies counted. This allowed the calculation of the ratio of mutant and wildtype organisms within the spleen.

At 48 hours post-challenge the competitive index was calculated as 0.69. However, when the titres of *B. pseudomallei* 576 and *B. pseudomallei* 6H2 recovered from spleens were

compared using regression analysis, the difference between the titres was not statistically significant (P value = 0.22).

6.5. Characterisation of the transposon mutant *B. pseudomallei* 6H2.

To identify the gene interrupted by the transposon in *B. pseudomallei* 6H2, genomic DNA was isolated (Section: 2.3.1.) and digested with the restriction endonuclease enzyme *Sac*1. The recognition site for this enzyme is absent from the miniTn5 transposon. Digested DNA was then cloned into the plasmid vector pUC18 which had been pre-digested with *Sac*1. Recombinant plasmids were transformed into *E. coli* XL10 Gold cells and plated onto LB-agar supplemented with kanamycin. Plasmids were isolated from kanamycin resistant clones and nucleotide sequenced using the primers M13 forward and M13 reverse (Appendix: 4.).

Sequence data obtained using the M13 forward primer was used to perform a blast search for homologus nucleotide sequences (BLASTn) in the National centre for Biotechnology Information (NCBI) sequence database. The sequence from the M13 forward primer matched the transposon delivery vector pUTKM1 a derivative of the miniTn5 transposon (Fang 2002) used to create the *B. pseudomallei* 576 transposon mutants (97% identity in 488 of 501 bases, E value 0.0).

Sequence data obtained using the pUC18 M13 reverse primer was subjected to a Blastn search using the Sanger centre online *B. pseudomallei* BLAST server (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/b_pseudomallei). This resulted an 89 % identity in 670 of 747 bases, from the 791 base sequence used in the query to a gene on chromosome II of the *B. pseudomallei* K96243 genome (E value 4.4e-124). The gene was

identified as putative member of the aldehyde dehydrogenase family with the accession number BPSS1466 (Figure: 6.5.).

The DNA sequence of the gene BPSS1466 was translated *in silico* using the DNAstar computer program Editseq version 5.05. This amino acid sequence was then used to perform a search of the Sanger institute protein families database of alignments and hidden Markov models (PFAM) (http://www.sanger.ac.uk/Software/Pfam/) for similarities to proteins of known structure and function. This resulted in a high scoring match (E value 1.1e-140) to proteins from the aldehyde dehydrogenase family.

Figure: 6.5. The location of the transposon in *B. pseudomallei* 6H2. The insertion was identified in a gene described as a member of the aldehyde dehydrogenase family with the accession number BPSS1466. Genes immediately upstream and downstream of BPSS1466 include BPSS1464 a putative transmembrane NADPH flavoprotein, BPSS1465 a putative thiamine biosynthesis lipoprotein precursor and BPSS1467 a putative aminotransferase protein. The approximate position of miniTn5 transposon insertion in BPSS1466 is shown in red.

6.6. Discussion.

The *C. elegans-Burkholderia* infection model described in Chapter: 4, was assessed for its usefulness in screening *B. pseudomallei* mutant strains for those displaying attenuated phenotypes. Initially a number of mutants that had previously been shown to be attenuated in mammals were screened using the plate-based mortality assay (Section: 6.2). This indicated that some mutants that were less virulent in mammalian models were also less virulent in nematodes.

The *B. pseudomallei* K96243 mutant *B. pseudomallei ΔpilA* was shown to have a reduced virulence towards *C. elegans* N2 in the plate-based mortality assay. As this mutant was also less virulent in mouse models of infection the product of the deleted gene must then be important in killing both mice and nematodes. The *pilA* gene is involved in the production of type IV pili in other organisms, such as *P. aeruginosa* and is also thought to be involved in type II secretion (Lu 1997). Hence the deletion of these genes may not only prevent the formation of type IV pili but also hinder secretion of virulence factors that could be important in killing of mammalian and nematode species by *B. pseudomallei.*

A number of *B. pseudomallei* 576 transposon mutants were also tested in *C. elegans* N2. No strain was attenuated indicating that not all mutants that have reduced virulence in mammals also have a decreased virulence towards nematodes. This would be expected, as all genes important in mammalian pathogenesis would not be useful in killing of nematodes due to the large phylogenetic differences observed between species.
For example the mutant *B. pseudomallei* 1E10 has a mutation in a biosynthetic gene responsible for the production of the polysaccharide capsule (Atkins 2002). This functions

in mammalian pathogenesis to inhibit phagocytosis and prevent killing of the bacterium by bactericidal serum components (Corbett 2003). Hence in mammals the mutant is less virulent as it has limited defences against these mechanisms. However, as *C. elegans* does not posses phagocytic cells such macrophages or have serum, the capsule is not required for protection and therefore the mutant is fully virulent in nematodes.

Over all this data suggests that genes important for virulence in both nematode and mammalian models of infection would be those that have a broad function, such as the secretion of virulence factors or the regulation of these processes. These could then be called "universal virulence factors", as they would allow an organism to be virulent in a variety of host species from different genera. These observations have already been reported in *P. aeruginosa* infection of different host species. A selection of genes were found to be important for pathogenesis of this organism in plants, nematodes and mice (Tan 2002) and thus the same may be the case for a set of *B. pseudomallei* genes such as *pilA*.

In this chapter the *C. elegans-Burkholderia* model was shown to be capable of identifying mutations that cause a decrease in virulence in nematodes and mammalian models of infection. This validated the use of the model for screening pools of mutants for those with an attenuated phenotype. Thus, the infection model was used to screen 960 *B. pseudomallei* 576 transposon mutants for mutations that affected virulence in nematodes. Initially 14 mutants were identified as having reduced virulence towards *C. elegans* N2 in a modified plate-based mortality assay. However, further investigation revealed that only one was less virulent when compared to the wildtype strain. Worms fed on *B. pseudomallei* 6H2 were found to exhibit a decreased rate of death in the plate-based mortality assay

(Figure: 6.3.) and an increased rate of feeding in the feeding inhibition assay (Figure: 6.4.) indicating a reduction in virulence of the mutant.

The results from the investigation of *B. pseudomallei* 6H2 in the plate-based mortality assay were similar to those obtained by other authors using similar assay methods to investigate *B. pseudomallei* mutants (Gan 2002). In these studies, attenuated *B. pseudomallei* mutants caused 100% death of nematodes. However worms were killed more slowly when compared to a fully virulent control. The mutants identified were subsequently found to also be attenuated in mammals. Hence the reduction in the rate of nematode killing was indicative of a reduction of virulence in mammalian models of infection (O' Quinn, Wiegand *et al.* 2001; Gan 2002). Thus, the reduction in the rate of nematode killing when fed on *B. pseudomallei* 6H2, compared to that seen on *B. pseudomallei* 576 may have also indicated that *B. pseudomallei* 6H2 was attenuated in mammals.

To assess this possibility the virulence of *B. pseudomallei* 6H2 and *B. pseudomallei* 576 was compared in BALB/c mice. This indicated that *B. pseudomallei* 6H2 was less virulent than the wildtype *B. pseudomallei* 576. The *in vivo* and *in vitro* competitive indices were also calculated. This revealed that the mutant *B. pseudomallei* 6H2 appeared to colonise the murine spleen less efficiently (competition index 0.69). The reduction in virulence of *B. pseudomallei* 6H2, observed in both nematode and mammalian models was small. However, overall the results indicated that *B. pseudomallei* 6H2 was less virulent than *B. pseudomallei* 576 in both these model systems. Thus, the *C. elegans-Burkholderia* infection model was found to be suitable for screening large groups of mutants for mutations that affect virulence in both nematodes and mammalian host species.

The interrupted gene in *B. pseudomallei* 6H2 was identified as a member of the aldehyde dehydrogenase family. How the interruption in this gene can result in the decrease in virulence observed for *B. pseudomallei* 6H2 is unclear. However in other organisms the role of these enzymes is the oxidation and detoxification of aldehydes to their corresponding carboxylic acid form, during the intracellular catabolism of some sugars (Xu 1995; Schrader 2001). Thus the inactivation of BPSS1466 might allow the build up of toxic intermediates, that cause a reduction in the ability of *B. pseudomallei* 6H2 to colonise *in vivo*.

Alternatively, this gene may play a role in the virulence of *B. pseudomallei* through other mechanisms as seen in *V. cholerae*. In this organism the gene *aldA* encodes a co-enzyme A dependent aldehyde dehydrogenase. Once thought to only function in metabolic pathways the enzyme is now thought to function in virulence through an unknown mechanism (Karaolis 1998). The gene *aldA* is found in a 39.5 kb pathogenicity island called VPI that includes other virulence related genes such as the toxin-co-regulated pilus, which is an essential colonisation factor in human and animal models of infection. Also adjacent to *aldA* and oppositely transcribed is the gene *tagA*, which encodes a lipoprotein. The presence of these genes has been associated with epidemic and pandemic strains of *V. cholerae* and thus they may have a role in virulence of this organism (Karaolis 1998). However, a definite role for this gene in the virulence of *V. cholerae* has never been determined. To my knowledge, this study represents the first occasion that the inactivation of an aldehyde dehydrogenase gene, from a bacterial pathogen has been shown to cause a decrease in virulence in either nematodes or mammals.

The possibility of the inactivation of BPSS1466 having polar effects on other genes that would result in a decrease in virulence must also be considered. Genes immediately

upstream and downstream of BPSS1466 include BPSS1464 a putative transmembrane NADPH flavoprotein, BPSS1465 a putative thiamine biosynthesis lipoprotein precursor and BPSS1467 a putative aminotransferase protein. These genes have roles in various essential metabolic processes within the bacterial cell such the catabolism of amino acids and the catalysis of various redox reactions (Stryer 1996; Hetti 2003). Thus interference with these genes may cause defects in growth both *in vitro* and *in vivo*.

A search of chromosome II, up and down-stream of BPSS1466 indicated that no genes previously recognised to be involved in virulence are present in this area of the genome. The region was composed of genes encoding putative metabolic and regulatory functions. Thus, polar effects on more distant genes that result in a decrease in virulence are also unlikely.

The net result of the interruption of the aldehyde dehydrogenase gene BPSS1466 in *B. pseudomallei* 576 was a reduction in virulence in the nematode *C. elegans* and in BALB/c mice. Further work, including the complementation of the interrupted gene in an attempt to restore wildtype levels of virulence, would be needed to determine a definite role for BPSS1466 in the virulence of *B. pseudomallei* 576.

In a similar study to that reported here Gan *et al* (Gan 2002) screened 3400 *B. pseudomallei* transposon mutants for those with reduced virulence in *C. elegans* N2. From these, 5 were isolated that were attenuated in nematodes and mice. Hence the success rate in the identification of attenuated mutants was 1 mutant in every 680 tested. The success rate for the screen performed here was 1 mutant in every 960 mutants. This suggests that the high-throughput screening method used in this study was not of comparable efficiency to the method reported by Gan. However a number of differences exist between the

methodology used in these studies that may account for the apparent reduction in efficiency. This includes differences in the growth media used, which has been shown to effect the kinetics of *B. pseudomallei-C. elegans* interactions (O' Quinn, Wiegand *et al.* 2001) and the use of a different *B. pseudomallei* strain for construction of the transposon library. Furthermore, approximately 3 times as many mutants were screened in Gan's study than in the study reported here. Therefore the possibility remains that through the screening of a comparable number of mutants a similar quantity displaying attenuated phenotypes may have been isolated. However, when the isolation rate of mutants displaying a reduced virulence in both nematodes and mammalian models is calculated, the isolation rate for Gan's study and the investigation reported here was the same at 0.1%. This indicates that both screening methods were of comparable efficiency thus validating the high through-put method used in this thesis.

The isolation rate of attenuated mutants from pools of transposon mutants in mammalian models ranges between 1-5% (Hensel, Shea *et al.* 1995; Chiang and Mekalanos 1998; Foulongne, Bourge *et al.* 2000; Fuller, Martin *et al.* 2000; Atkins 2002). As discussed above the use of a nematode model of infection for isolation of mutants attenuated in mice is less efficient. This would be expected as when using a mammalian model to identify mutations that cause a reduction of virulence in mammals, only genes that reduce virulence in one animal species is considered. However, when using the *C. elegans* model, genes that attenuate virulence in two different animal species must be identified. Thus the probability of isolating a single gene that reduces virulence in both nematodes and mice would be less, hence the difference in efficiency shown between screens in mammalian and worm models of infection.

Although shown to be less effective, screens using *C. elegans* for the isolation of *B. pseudomallei* mutants that are attenuated in mammals have been successful. Moreover the genes identified in such screens are often not previously thought to function in virulence (Gan 2002). Thus the use of the nematode model of infection in these applications may provide valuable information regarding the virulence of *B. pseudomallei* that would not be obtainable in a conventional mammalian model system.

Chapter: 7. Identification of *B. pseudomallei* virulence factors using cosmid libraries.

7.1. Introduction.

The work presented in chapter: 6 showed that the *C. elegans-B. pseudomallei* infection model could be used to identify *B. pseudomallei* mutants with reduced virulence. This prompted an investigation of other molecular methods for the identification of bacterial virulence factors. Cosmid libraries, carrying fragments of genomic DNA from a pathogen of interest, can be expressed in a non-pathogenic host such as *E. coli*. Virulence related genes contained within the cloned DNA of cosmids can then be identified through the increase in virulence of the host strain. This approach has been used successfully for the identification of virulence genes in *S. enterica* serovar Typhimurium (Robey 2002). A cosmid library was constructed from fragments of the genome of a fully virulent and invasive strain of this organism. The library was then introduced into an avirulent strain and the resulting clones screened for intestinal invasiveness in a rabbit ileal loop assay (Robey 2002). This resulted in the isolation of one recombinant strain that had increased invasiveness compared to a control. The cloned DNA fragment responsible for the increase in virulence was sequenced and mapped to a region of the *S. enterica* serover Typhimurim chromosome close to the *Salmonella* pathogenicty island 5 (SPI-5) (Robey 2002}. The cloned genes are thought to encode pleiotrpic regulators of genes involved in gastro-enteric virulence and adaptation to the *in vivo* gut environment. This work suggested that virulence-associated genes from other bacterial species could be identified using a similar method. Hence the usefulness of the *C. elegans* infection model for the isolation of cosmid clones carrying fragments of *B. pseudomallei* DNA with an increased virulence was assessed.

7.2. Screening of a *B. pseudomallei* E504 cosmid library using *C. elegans* N2.

A *B. pseudomallei* E504 cosmid library created using the cosmid vector supercos-1 (Stratagene, UK Appendix: 1.) was obtained in *E. coli* XL1-blue MR (Stratagene, UK) from Dr. C. Winstanley, Department of medical microbiology and GU medicine, University of Liverpool. On receipt, the library was validated for the presence of different cloned sequences. An overnight culture of the *B. pseudomallei* E504 cosmid library was diluted and plated onto media containing ampicilin, to obtain single colonies. Ten colonies were selected and cosmid isolated from overnight cultures of each clone using a Quiagen miniprep kit. The vector was then digested overnight with the restriction endonuclease enzyme *Not* 1. The multiple cloning site of the vector supercos-1 is flanked by unique *Not* 1 restriction enzyme recognition sites (Appendix: 1.). This allowed the excision of cloned DNA fragments while leaving the vector intact. From the 10 *E. coli* clones selected, all were shown to carry the supercos-1 vector and 9 were found to have DNA fragments. However, one clone was shown to contain no inserted sequence and only supercos-1 was present. Thus, approximately 90 % of the cosmid library could be expected to contain different cloned *B. pseudomallei* DNA fragments (Figure: 7.1).

To serve as a control in subsequent experiments, an *E. coli* XL1-Blue MR strain was transformed with empty supercos-1 cosmid vector (Section: 2.3.7.). This strain was named *E. coli* XL:cos-1 (Figure: 7.1).

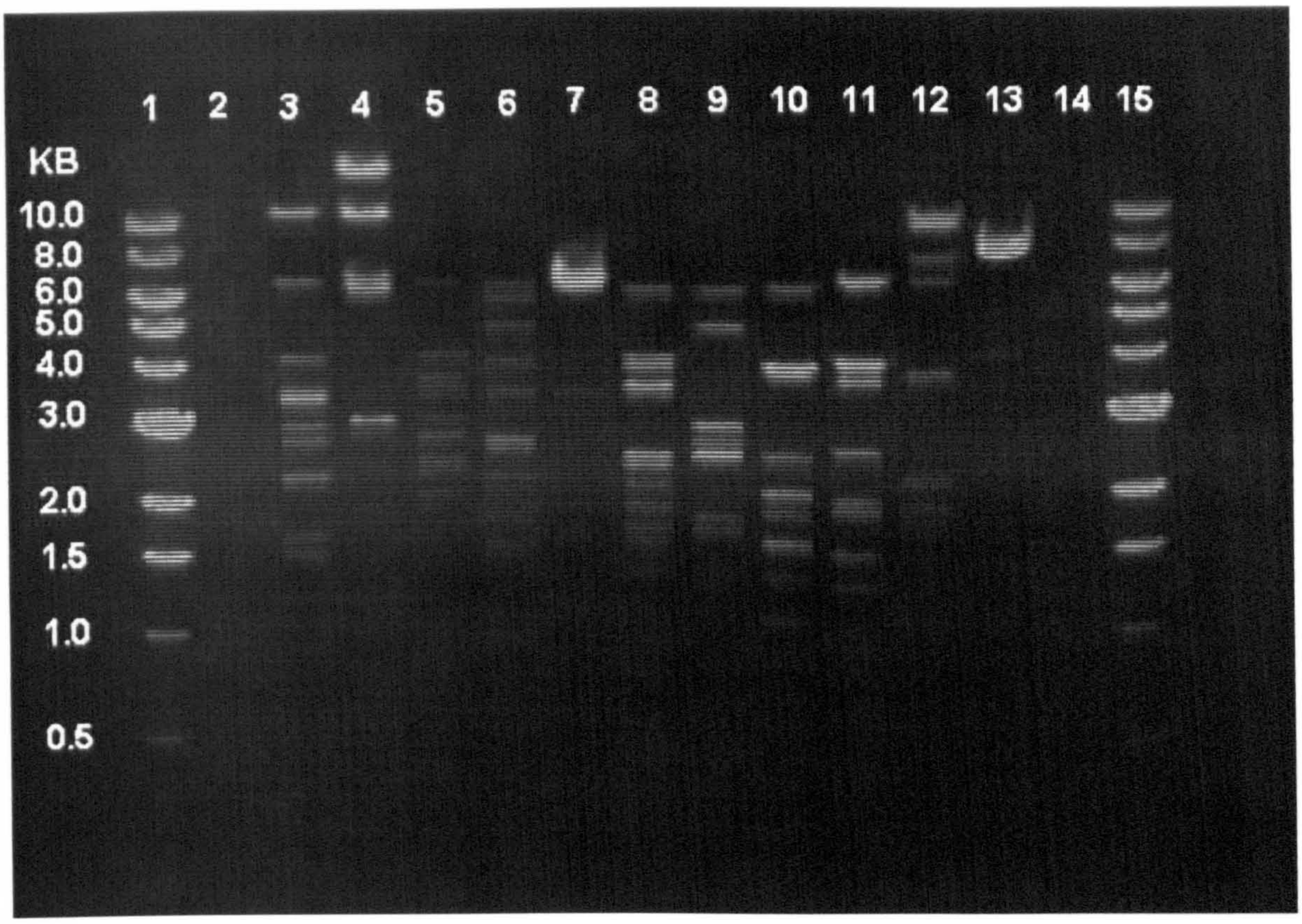


Figure: 7.1: Agarose gel showing DNA isolated from randomly selected *E. coli* XL1-Blue MR clones digested with the restriction enzyme *Not* 1. Cosmid was extracted from 10 randomly selected clones and digested with the restriction endonuclease *Not* 1. This was compared to supercos-1 supplied by the manufacturer (Stratagene. UK) through agarose gel electrophoresis at 80v. Lanes 1 and 15 molecular mass markers, lanes 3-12 digests and lane 13 contains supercos-1.

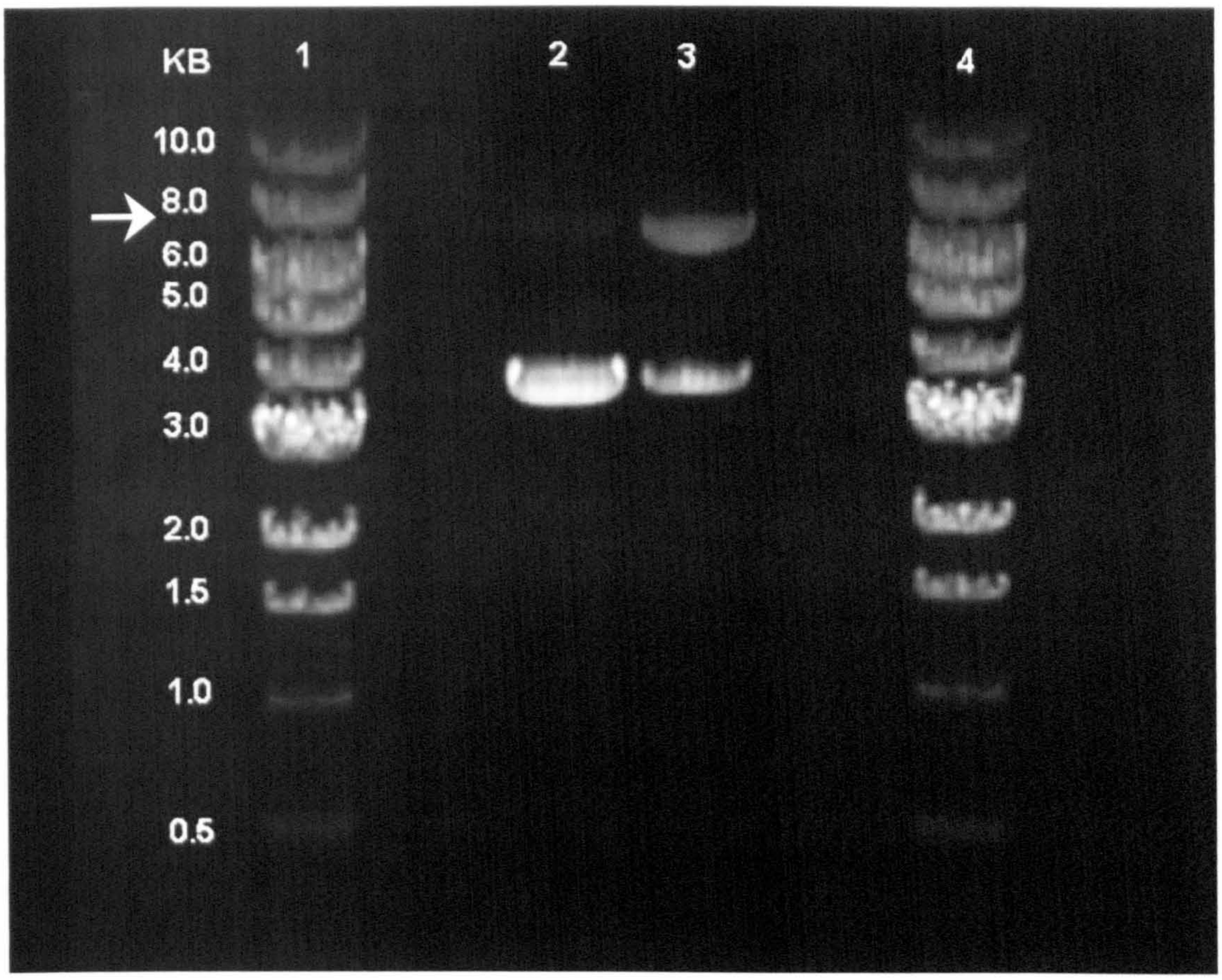


Figure: 7.2. Comparison of purified supercos-1 cosmid vector (Stratagene, UK) supplied by the manufacturer (lane 2) and supercos-1 cosmid vector extracted from transformed *E. coli* XL1-blue cells (lane 3) on a 0.7 % agarose gel at 80v. The white arrow denotes a 7.9 kb band corresponding to the circular form of supercos-1. A band can also be seen with an approximate size of 4.0 kb, which represents the supercoiled form of the vector.

An overnight culture of the *B. pseudomallei* E504 cosmid library in *E. coli* XL-1 Blue MR was diluted and plated onto media containing ampicilin, to obtain single colonies. A selection of 150 cosmid clones were then chosen and investigated for their effect on feeding rates of *C. elegans* N2 in a feeding inhibition assay (Section: 2. 4.6.).

Groups of 100-200 *C. elegans* N2 nematodes were fed in suspensions of each clone at an approximate starting OD_{550nm} of 1.2 at 25 °C for a total 48 hours. The OD_{550nm} of each suspension was recorded at the beginning of the experiment and at 48 hours. Clones were said to have a negative effect on nematode feeding if results after conversion into AUCD were less than the average of all of the clones minus 2.25 times the standard deviation of all the results obtained for the other clones. This ensured a high degree of confidence that any clone identified significantly inhibited feeding.

After screening all 150 cosmid clones, one clone (clone 93) was found that gave a negative effect on feeding rate of *C. elegans* N2 according to the criteria described above. To assess the degree of the feeding inhibition induced, clone 93 was investigated further in the feeding inhibition assay over 48 hours.

Groups of 50- 100 *C. elegans* N2 worms were fed on suspensions of clone 93 with an approximate starting OD_{550nm} of 1.2, for 48 hours at 25 °C, and the OD_{550nm} monitored at daily intervals (Figure: 7.3). At 48 hours, consumption rates on clone 93 and XL-cos-1 were 0.46 AUCD and 0.3 AUCD respectively. Hence feeding was faster in suspensions of clone 93. This indicated no-inhibition of nematode feeding by the clone and therefore this strain was not investigated further.

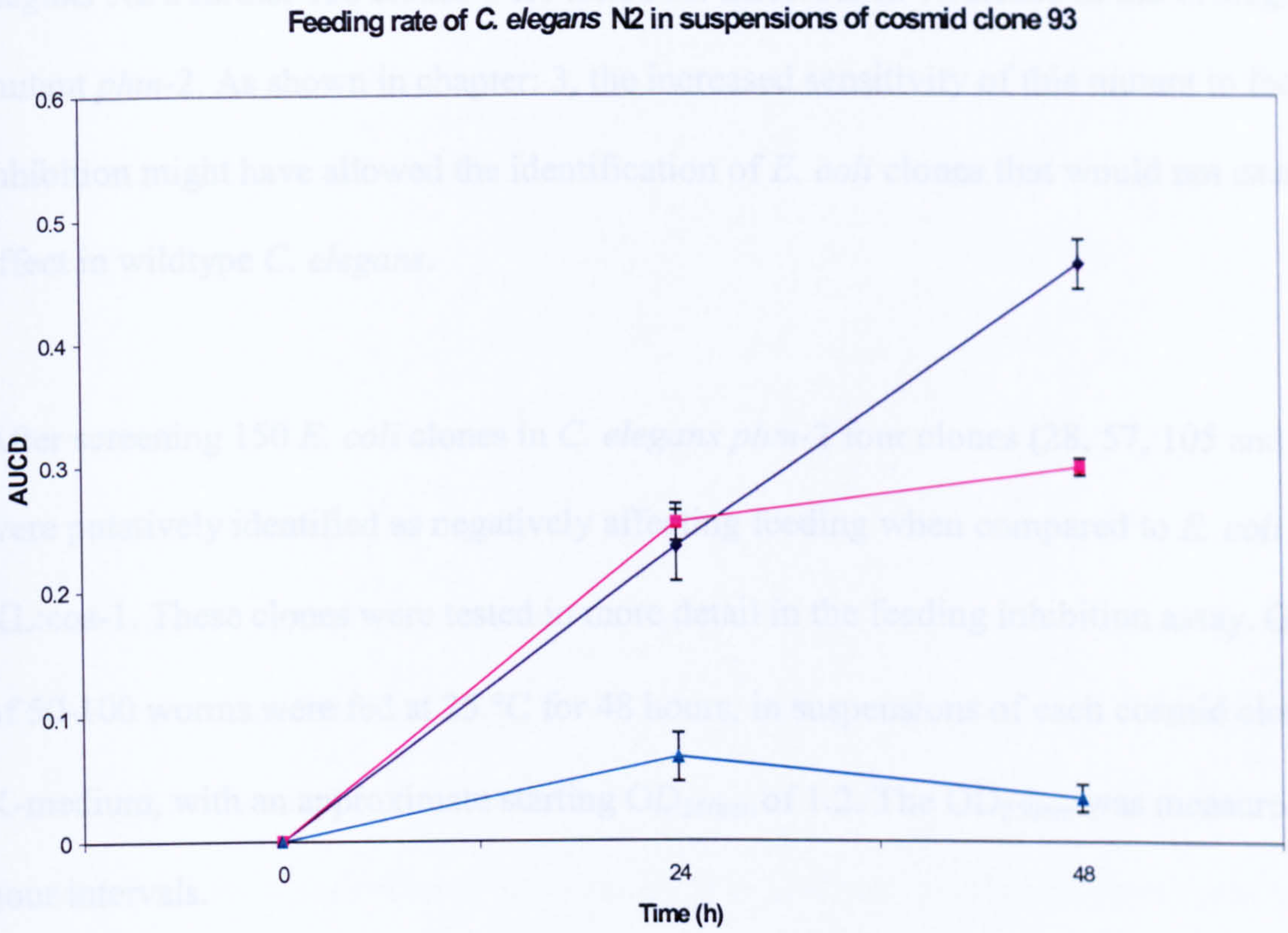


Figure: 7.3. Feeding inhibition of *C. elegans* N2 at 25 °C over 48 hours in suspensions of clone number 93 (◆) in K-medium with an approximate stating OD_{550nm} of 1.2. Feeding in suspensions of *E. coli* XL:cos-1 (■) or XL:cos1 with the addition of 100 mM sodium salicylate (▲) were used as controls. Error bars are the standard error of the mean of 8 replicates performed for each condition each containing 50-100 L4 stage worms.

7.3. Screening of a *B. pseudomallei* E504 cosmid library using *C. elegans phm*-2.

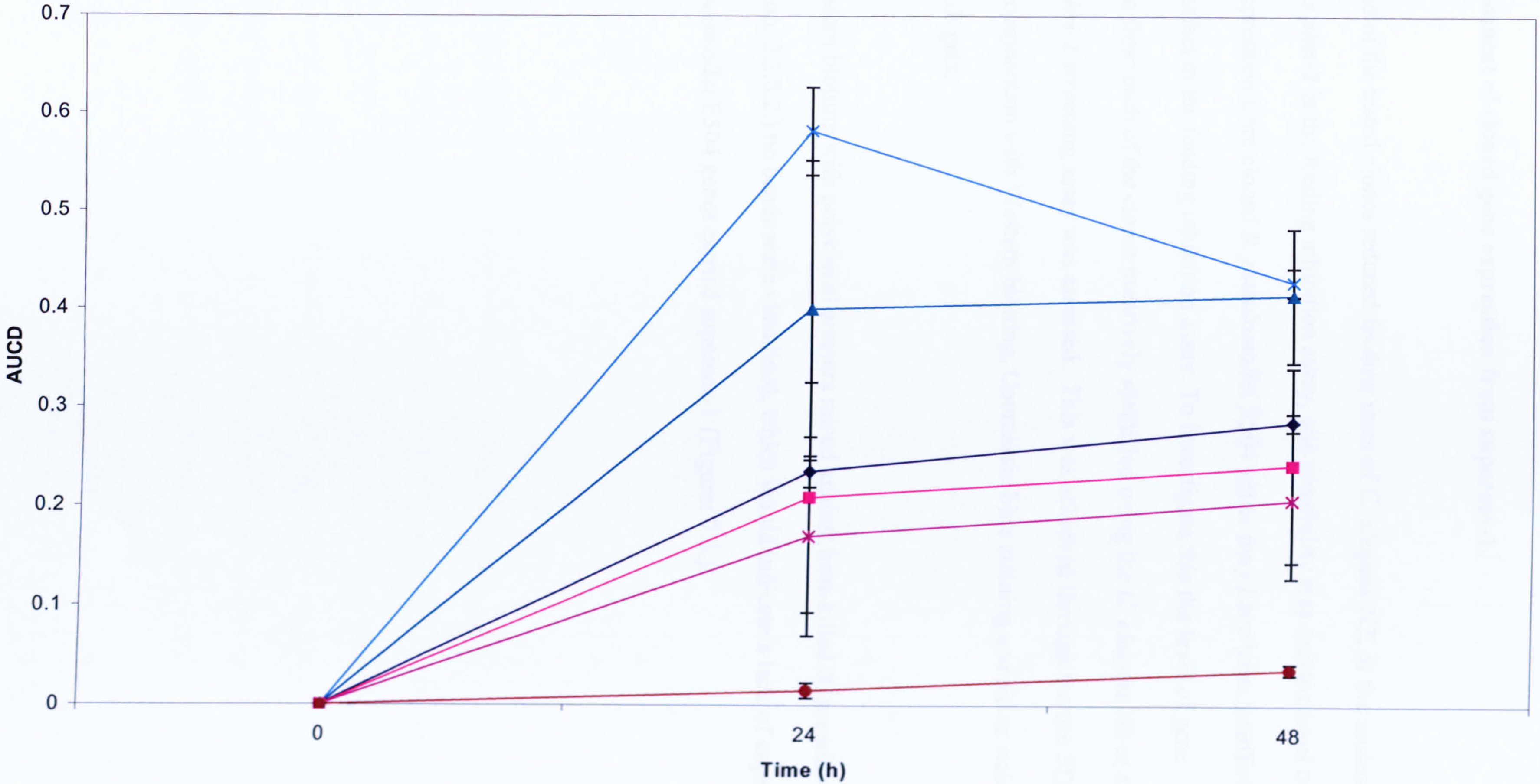
Since no cosmid clones were identified as having a detrimental effect on feeding in *C. elegans* N2 a further 150 clones were tested for increases in virulence in the *C. elegans* mutant *phm*-2. As shown in chapter: 3, the increased sensitivity of this mutant to feeding inhibition might have allowed the identification of *E. coli* clones that would not cause an effect in wildtype *C. elegans*.

After screening 150 *E. coli* clones in *C. elegans phm*-2 four clones (28, 57, 105 and 119) were putatively identified as negatively affecting feeding when compared to *E. coli* XL:cos-1. These clones were tested in more detail in the feeding inhibition assay. Groups of 50-100 worms were fed at 25 °C for 48 hours, in suspensions of each cosmid clone in K-medium, with an approximate starting OD_{550nm} of 1.2. The OD_{550nm} was measured at 24 hour intervals.

After testing all four *E. coli* clones in the feeding inhibition assay no strain was seen to significantly reduce feeding when compared to feeding rates observed on *E. coli* XL:cos-1 (Figure: 7.4.).

Figure: 7.4. Feeding rates of *C. elegans phm*-2 at 25 °C over 48 hours while feeding in suspensions of *E. coli* clones number 28 (◆), 57 (■), 105 (▲) or 119 (✕) in K-medium with an approximate stating OD_{550nm} of 1.2. Feeding in suspensions of *E. coli* XL:cos-1 (✳) and XL:cos1 with the addition of 100 mM sodium salicylate (●) were used as controls. Error bars are the standard error of the mean of 8 replicates performed each containing 50-100 L4 stage worms.

Feeding rates of *C. elegans phm*-2 on *E. coli* cosmid clones.



7.4. Assessment of cloned gene expression from supercos-1.

Since none of the tested clones reduced feeding rates of *C. elegans* N2, or the mutant strain *C. elegans phm*-2 in the feeding inhibition assay, one possibility was that the level of protein expression from cloned *B. pseudomallei* E504 genes may have been insufficient to cause an effect in the feeding inhibition assay. To investigate this the level of gene expression from each of the clones putatively identified using the *C. elegans* N2 or *C. elegans phm*-2 screening assay was assessed. This was achieved through the use SDS-PAGE in conjunction with Western blotting, Coomassie blue staining and silver staining of SDS-PAGE gels.

After Western blotting with polyclonal antisera raised against heat-killed *B. pseudomallei* 576 (section: 2.3.8.2.) no bands were visualised, which would indicate a lack of expression of *B. pseudomallei* E504 genes carried supercos-1 (Figure: 7.5.).

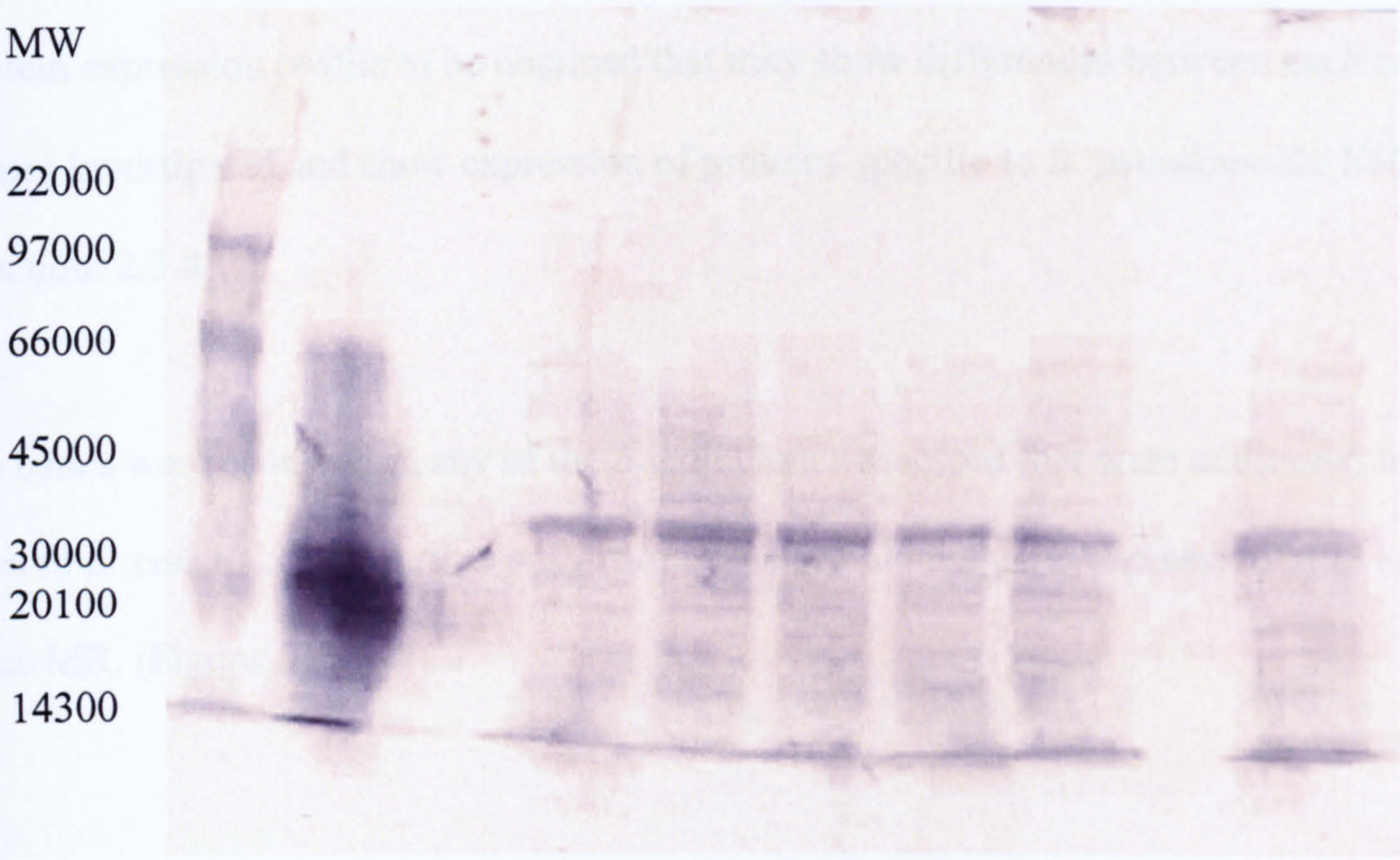


Figure: 7.5. Western blot of an SDS-PAGE gel containing boiled samples of *E. coli* clones previously putatively identified as having a negative effect on feeding rates in the feeding inhibition assay on *C. elegans* N2 and the mutant *C. elegans phm*-2. Developed membranes were visualised and recorded using a Multigenius Bio-imaging system (Syngene, Cambridge, UK). To give an indication of molecular weight samples were ran against a high range rainbow molecular weight marker (Amersham, UK).

To further assess levels of expression of cosmid inserts from each clone, SDS-PAGE gels were stained using Coomassie brilliant blue. This method allows the visualisation of all proteins contained within samples and avoids many of the problems of "background noise" experienced with Western blotting (Gooderham 1984). This then would allow a more clear protein expression profile to be obtained that may show differences between each of the clones investigated and show expression of proteins specific to *B. pseudomallei* E504 (Section: 2.3.8.1.).

No bands were observed in any of the *E. coli* clones assessed that were additional to those seen in *E. coli* XL:cos-1 further suggesting a lack of cloned gene expression in *E. coli* XL1 Blue MR. (Figure: 7.6.).

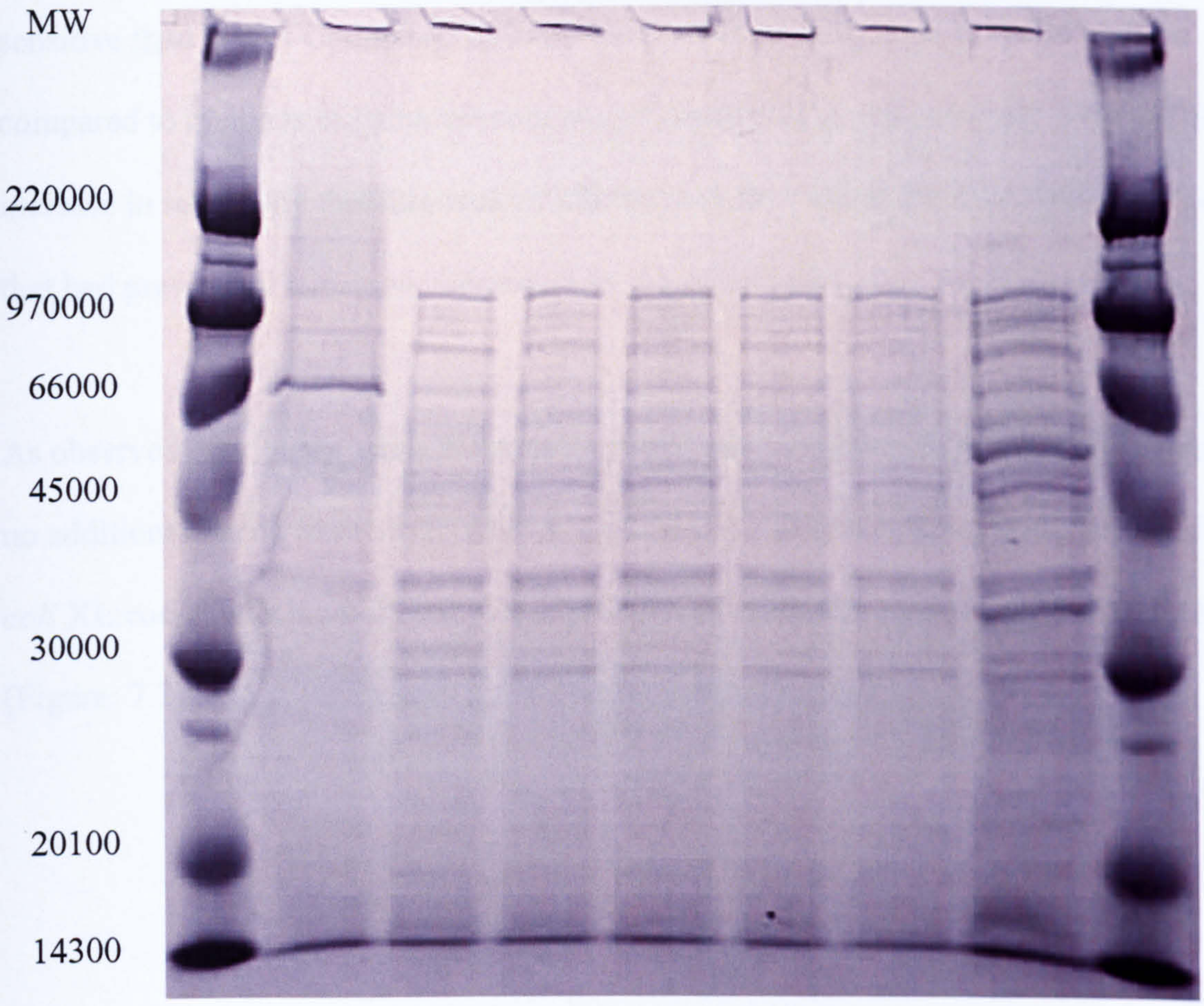


Figure: 7.6. Coomassie stain of an SDS-PAGE gel containing boiled samples of cosmid clones previously identified as having a negative effect on feeding rates in the feeding inhibition assay on *C. elegans* N2 and the mutant *C. elegans phm*-2. Gels were visualised and recorded using a Multigenius Bio-imaging system (Syngene, Cambridge, UK). To give an indication of molecular weight samples were ran against a high range rainbow molecular weight marker (Amersham, UK).

Finally, SDS-PAGE gels were subjected to silver staining to visualise proteins after electrophoresis. Silver staining of proteins is considered to be 20 and 200 times more sensitive than that of Coomassie brilliant blue, which is of limited sensitivity when compared to methods utilising silver containing compounds (Gooderham 1984). The increase in sensitivity that this method affords may then allow the visualisation of proteins that had previously remained undetected by the other techniques so far employed.

As observed previously using Western-blotting and Coomassie staining of SDS-PAGE gels no additional bands were observed in any of the cosmid clones investigated compared to *E. coli* XL:cos-1, which would indicate expression of cloned *B. pseudomallei* E504 genes (Figure: 7.7).

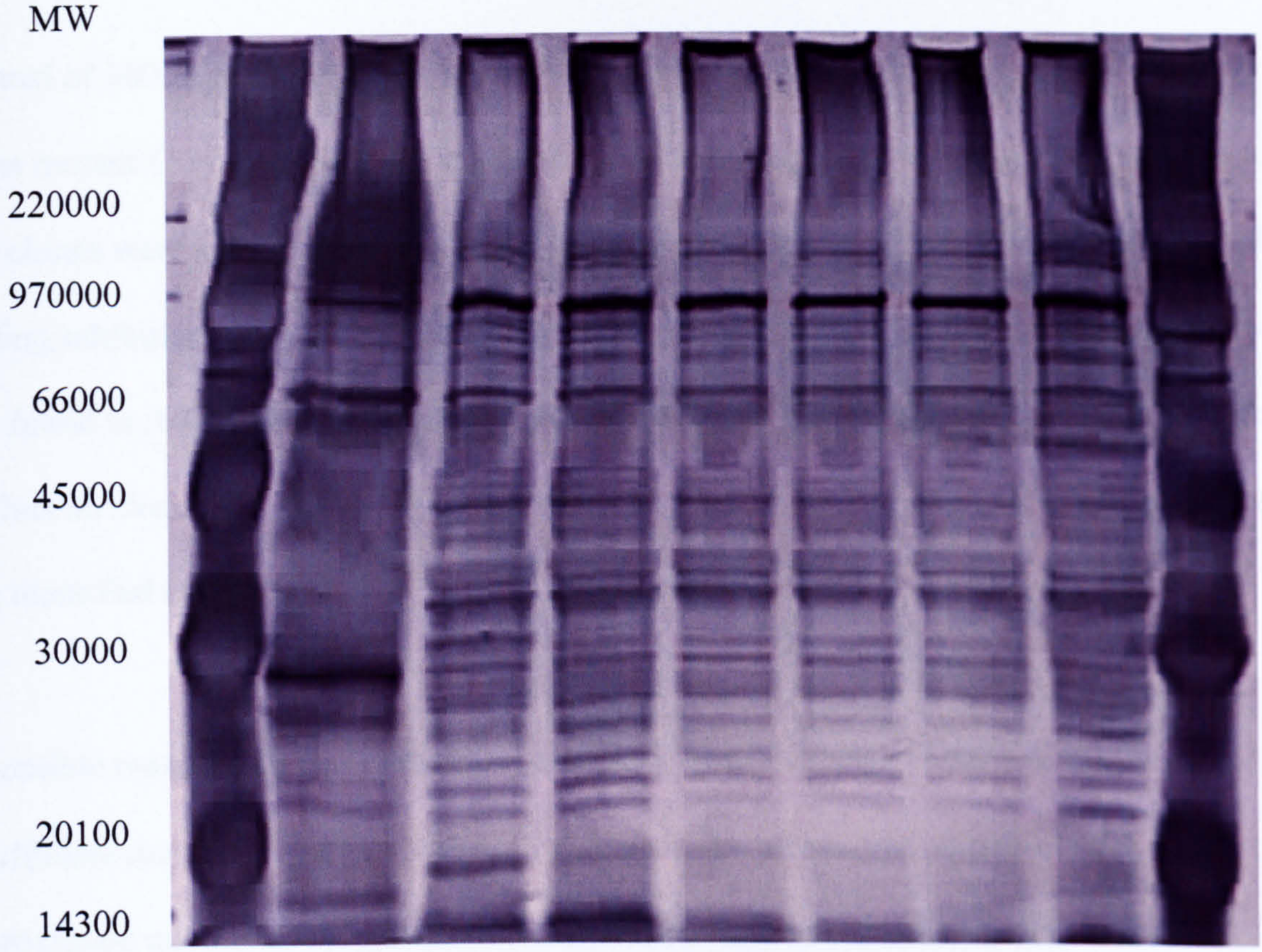


Figure: 7.7. Silver stain of an SDS-PAGE gel containing boiled samples of cosmid clones previously identified as having a negative effect on feeding rates in *C. elegans* N2 and the mutant *C. elegans phm*-2. Gels were visualised and recorded using a Multigenius Bio-imaging system (Syngene, Cambridge, UK). To give an indication of molecular weight samples were ran against a high range rainbow molecular weight marker (Amersham, UK).

7.5. Discussion.

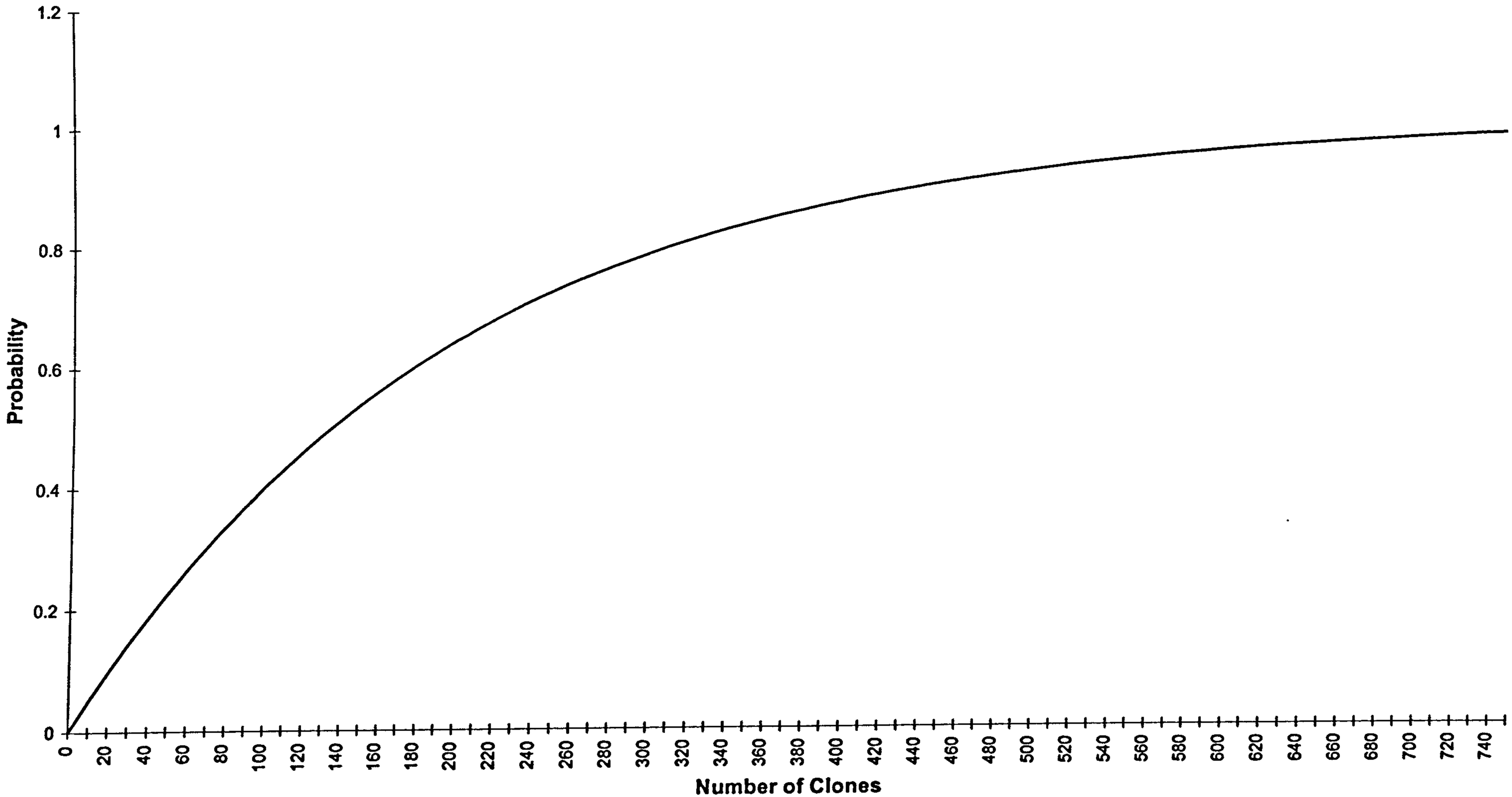
A panel of 300 *B. pseudomallei* E504 cosmid clones were screened in either *C. elegans* N2 or the mutant *C. elegans phm*-2 for increased pathogenicity phenotypes. From these a total of 5 clones were putatively identified as having a detrimental effect on feeding rate in the feeding inhibition assay. However on analysis using the feeding inhibition assay no clone was found to reduce feeding rates to below that observed in suspensions of *E. coli* XL:cos-1. Thus no clone exhibiting an increase in virulence in the *C. elegans* model of infection was identified in this study.

A possible reason why no clones with increased virulence were identified may be that insufficient numbers of clones had been screened. To assess this possibility the approximate number of clones needed to be tested in the nematode model, to ensure complete coverage of the *B. pseudomallei* E504 genome was calculated through the use of the Clarke and Carbon formula. That is $N = \ln (1 - P) / \ln (1 - 1/n)$ where P is the probability a given DNA sequence is present, n is the ratio of insert size to genome size and N is the number of clones needed to ensure each sequence of a particular size is represented at least once in a panel of clones (Medhora, Phadnis *et al.* 1983). *B. pseudomallei* has approximate genome size of 7 Mb (http://www.sanger.ac.uk/Projects/B_pseudomallei/) and the supercos-1 cosmid vector has an average insert size of approximately 36 kb (http://www.stratagene.com/displayProduct.asp?productId=275). Therefore for a 99·% chance of screening the entire genome for genes that give rise to an increase in virulence an estimated 892 clones, all containing cloned sequences, would be needed to be tested in nematodes (Figure: 7.7.). A total of only 300 clones were screened in either *C. elegans* N2

or *C. elegans phm*-2, giving only a 78 % probability of the whole genome being represented a least once in the form of 36 kb segments. The fact that only 90 % of the cosmid library were expected to contain cloned fragments of DNA must also be taken into account. Thus, the probability of complete coverage was reduced by a further 10 % to 68%. This indicates that the sample size used may not have been sufficiently large enough and that there remains a probability that some regions of the genome would have been missed from the screen. This may then account for the lack of clones identified with increased virulence in *C. elegans*.

Figure: 7.8. Probability of whole *B. pseudomallei* E504 genome coverage using the cosmid vector supercos-1 (Stratagene, UK). Values were calculated through the use of the Clarke and Carbon formula $N = \ln (1 - P) / \ln (1 - 1/n)$. This assumes a genome size of 7 Mb, an average supercos-1 insert size of 36 kb and a 99 % confidence of complete genome coverage.

Probability of whole *B. pseudomallei* E504 genome coverage using the cosmid vector Supercos-1.



A second possibility of why no effect was seen in the nematode model may due to the size of sequences that can be cloned into the supercos-1 vector. Bacterial virulence factors such as the TTS system (Cornelis 2002) and adhesisns are formed from different components encoded by genes organised into large operons. The upper size limit for cloned sequences in supercos-1 is 42 kb (http://www.stratagene.com/displayProduct.asp?productId=275). Thus functional virulence factors consisting of large co-ordinated collections of genes could not be cloned into supercos-1 due to the size limitations imposed by the multiple cloning site. Further more, these structures must be assembled correctly and localised in the outer membrane. This often requires the use of chaperone molecules and secretory signals that may not be present in the cloned sequence (Cornelis 2002). More over the regulatory mechanisms needed to control the production of these structures may also be absent. Thus, it is likely that fragments of such operons, consisting of single or few genes would be cloned. Hence fully functional virulence factors may not be produced and would have no effect towards *C. elegans* in the feeding inhibition assay.

A further possibility is that levels of expression of protein from the cloned sequences may be very low. This is supported by the fact that no expression of *B. pseudomallei* protein was detected in any clones investigated through Western–blotting, Coomassie staining or silver staining of SDS-PAGE gels. This suggests that amounts of protein expressed from cloned sequences in supercos-1 were below the detection level of these methods.

The transcription and translation of proteins from other bacterial species in *E. coli* is often hampered by problems inherent when using *E. coli* host cells to express foreign genes. For example codon usage at the level of translation may present a problem due to codon bias. Often a number of different codons can encode the same amino acid (Brown 1994). However, particular codons may be more important in protein translation in different

bacterial species due to their relative abundance within the cell. For example, one of the four codons that encode the amino acid alanine is GCG (Table: 7.1.a.). In *E. coli* K12 the frequency of this codon per thousand codons is 29.2 (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=Escherichia+coli+[gbbct]). However, in *B. pseudomallei* K96243 (Table: 7.1.b.) the frequency of the same codon is 80.0 (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=Burkholderia+pseudomallei+[gbbct]) indicating that this codon is used more frequently and is more important in *B. pseudomallei*. Hence proteins that contain alanine, encoded by genes containing the GCG codon would not be expressed efficiently when introduced into *E. coli* K12. This would be due to the relative lack of the corresponding tRNA molecules needed to form the protein from the codons present within the host cell. Thus the net result would be inefficient translation of the *B. pseudomallei* gene.

UUU 22.2(82893)	UCU 10.8(40338)	UAU 17.8(66278)	UGU 5.2(19594)
UUC 15.9(59326)	UCC 9.3(34896)	UAC 12.1(45113)	UGC 6.0(22429)
UUA 14.4(53828)	UCA 9.4(35189)	UAA 2.0(7362)	UGA 1.0(3821)
UUG 12.8(47667)	UCG 8.5(31755)	UAG 0.3(1060)	UGG 13.8(51464)
CUU 12.1(45176)	CCU 7.6(28496)	CAU 12.5(46664)	CGU 19.6(73065)
CUC 10.1(37754)	CCC 5.4(20205)	CAC 9.1(34072)	CGC 19.1(71169)
CUA 4.3(16072)	CCA 8.6(32003)	CAA 14.4(53581)	CGA 3.9(14537)
CUG 47.4(177073)	CCG 20.1(75226)	CAG 28.4(106132)	CGG 6.2(23004)
AUU 29.6(110665)	ACU 10.8(40452)	AAU 21.4(79985)	AGU 10.3(38461)
AUC 23.3(87013)	ACC 21.8(81518)	AAC 21.5(80233)	AGC 15.0(56137)
AUA 7.5(27837)	ACA 10.0(37331)	AAA 35.2(131606)	AGA 4.0(14945)
AUG 26.0(97214)	ACG 13.7(51308)	AAG 12.8(47643)	AGG 2.3(8675)
GUU 20.0(74656)	GCU 17.4(64843)	GAU 32.8(122564)	GGU 25.3(94598)
GUC 14.2(53076)	GCC 24.1(89946)	GAC 19.2(71549)	GGC 26.5(98914)
GUA 11.7(43741)	GCA 21.4(79944)	GAA 38.5(143639)	GGA 10.0(37357)
GUG 23.9(89076)	GCG 29.2(108870)	GAG 18.7(69898)	GGG 11.4(42715)

Table: 7.1. a. *E. coli* K12 codon usage. The fields shown are; the three letter triplet corresponding to a particular amino acid, the frequency of that codon per thousand codons and finally the number of that particular codon per cell is shown in parentheses.

UUU 6.8(366)	UCU 2.1(115)	UAU 9.6(513)	UGU 1.5(79)
UUC 28.7(1539)	UCC 8.1(435)	UAC 14.4(769)	UGC 7.7(412)
UUA 0.9(46)	UCA 2.1(110)	UAA 0.6(31)	UGA 2.0(105)
UUG 10.7(575)	UCG 29.4(1577)	UAG 0.3(17)	UGG 13.3(711)
CUU 6.7(360)	CCU 2.8(148)	CAU 8.1(435)	CGU 7.6(405)
CUC 36.9(1976)	CCC 9.7(521)	CAC 12.2(654)	CGC 41.8(2238)
CUA 1.6(86)	CCA 1.9(103)	CAA 9.6(515)	CGA 5.7(303)
CUG 43.8(2347)	CCG 33.0(1769)	CAG 27.3(1465)	CGG 15.3(818)
AUU 8.0(427)	ACU 2.0(107)	AAU 8.3(445)	AGU 2.3(125)
AUC 38.0(2036)	ACC 14.8(794)	AAC 22.8(1221)	AGC 16.2(867)
AUA 1.9(104)	ACA 2.5(132)	AAA 5.9(314)	AGA 1.5(82)
AUG 22.5(1206)	ACG 34.8(1867)	AAG 24.7(1324)	AGG 2.7(144)
GUU 6.3(335)	GCU 6.6(352)	GAU 18.5(992)	GGU 6.0(321)
GUC 32.0(1717)	GCC 33.5(1795)	GAC 36.1(1932)	GGC 56.6(3033)
GUA 3.8(205)	GCA 11.2(602)	GAA 19.8(1063)	GGA 5.6(300)
GUG 32.5(1742)	GCG 80.0(4284)	GAG 29.4(1576)	GGG 11.0(587)

Table: 7.1.b. *B. pseudomallei* codon usage. The fields shown are; the three letter triplet corresponding to a particular amino acid, the frequency of that codon per thousand codons and finally the number of that particular codon per cell is shown in parentheses.

If correctly translated by an *E. coli* host strain a further obstacle to the correct function of many proteins is that some may require covalent processing through post-translational modification to become active or reach their correct localisation within the cell (Mann 2003). These modifications change the properties of a protein by proteolytic cleavage or addition of a modifying group to one or more amino acids. Common post-translational modifications include phosphorylation, which may activate or inactivate enzymatic activity or modulate molecular interactions and glycosylation that may be used in the stabilisation of excreted proteins and in cell-to-cell signaling (Mann 2003).

Some bacterial toxins including diphtheria toxin must undergo post-translational modification before becoming fully active. Diphtheria toxin is produced as a single peptide that must be proteolytically cleaved to form the A and B subunits necessary for toxic activity. Without this modification no toxic activity is seen (Middlebrook and Dorland 1984). Hence protein translated from putative *B. pseudomallei* virulence genes, expressed *in E. coli* may not function due to a lack of essential post-translational modifications that confer full activity.

In this study the search for *E. coli* clones with an increased virulence using the *C. elegans* model of infection has not proven effective. However levels of protein expression may be insufficient to cause an affect in this assay. Hence a further study utilising a *B. pseudomallei* E504 cosmid library, re-packaged and expressed in host cells where possibilities of codon bias and other factors that encumber protein expression are minimised, such as BL21-codon plus (Stratagene, UK) is needed. These host cells have been specially engineered to express codons suitable for the expression of GC-rich genomes thereby ameliorating problems of codon bias that may be experienced. This may

help to increase the level of *B. pseudomallei* E504 transcript present and allow the identification of clones with increased virulence using the feeding inhibition assay.

Chapter: 8. Discussion

8.1. A nematode model of bacterial infection using *C. elegans*.

The aim of the work reported in this thesis was to develop and exploit techniques for the investigation of bacterial pathogenicity using the nematode *C. elegans*. A *C. elegans*-*Burkholderia* infection model was used to investigate the virulence of a range of *Burkholderia* strains. The cellular interactions that occur in the *C. elegans* gut during infection with *B. pseudomallei* were studied using TEM. Two molecular approaches for the study of bacterial virulence were also investigated; the screening of *B. pseudomallei* transposon mutants for reduced virulence and the screening of a genomic cosmid library for clones that displayed an increased virulence in the worm model of infection.

Initially, a plate-based mortality assay was established on NGM media using the bacterial pathogen *P. aeruginosa* PA14 as a model of bacterial infection (Tan, Rahme *et al.* 1999)(Chapter. 3.). The results from the use of this assay closely resembled those published by other authors using similar assays (Tan, Rahme *et al.* 1999; Rahme, Ausubel *et al.* 2000; Yorgey, Rahme *et al.* 2001; Aballay and Ausubel 2002; Ewbank 2002). Similar plate-based mortality assays have been used for the study of other human pathogens including *S. marcescens*, (Pujol, Link *et al.* 2001) *S. enterica* serovar *Typhimurium* (Labrousse, Chauvet *et al.* 2000), *E. faecalis*, *S. pneumoniae* and *S. aureus* (Garsin, Sifri *et al.* 2001). This has lead to the identification of a variety of genes not previously known to be involved in the virulence of these organisms. Data from previous reports combined with the results presented here indicated that the plate-based mortality assay described in chapter: 3 could also be used as a tool for the investigation of bacterial virulence.

A novel method for the measurement of bacterial virulence was developed using a feeding inhibition assay. This assay was based on previous observations by Jones and Candido (Jones and Candido 1999) of a behavioural response in *C. elegans* that resulted in an inhibition of feeding in a bacterial suspension, when worms were stressed by heat or chemicals. Feeding inhibition could be monitored by measuring the OD_{550nm} of the bacterial suspension in which the nematodes feed, over time. The feeding inhibition assay was used to assess the virulence of *P. aeruginosa* towards *C. elegans* and this assay was found to be more rapid than the plate-based mortality assay.

A *C. elegans-Burkholderia* infection model was used to investigate a range of strains from the *Burkholderia* genus. This indicated that *C. elegans* was susceptible to infection by strains from the *B. cepacia* complex, *B. thailandensis* and *B. pseudomallei*. Those *B. pseudomallei* strains reported to have a reduced virulence in mammals also had a reduced virulence in nematodes. Importantly the investigation of these *B. pseudomallei* strains also showed that the feeding inhibition assay might not be as useful for the investigation of bacterial virulence using *C. elegans* as the plate-based mortality assay.

Using the plate-based mortality assay, the killing rates of each *B. cepacia* complex strain was shown to be different. No one genomovar had more nematode-virulent strains than another. Strains previously described as causing severe disease in humans were not more virulent than other strains in *C. elegans*. Some strains caused nematodes to move aberrantly. One strain, *B. cenocepacia sp. nov.* J415, also induced the transient formation of nose-globs. The pathogen *Y. pseudotuberculosis* has also been shown to form these adherent structures on the anterior and posterior cuticular surfaces of *C. elegans*. However nose-glob formation by *B. cenocepacia sp. nov.* J415 occurred through a mechanism distinct from that employed by *Y. pseudotuberculosis*. *B. cenocepacia sp. nov.* J415 is the

index strain for the degenerative condition cepacia syndrome in CF patients (Dr. J. Govan, Pers. Comm.). This strain may have adaptations for colonisation of the CF lung which also allow it to induce the formation of nose-globs in *C. elegans*. Overall, the link between aberrant movement and nose-glob phenotypes in nematodes, and pathogenicity of *B. cenocepacia sp.nov* J415 in the mammalian host, remains unclear.

When *B. cepacia* complex strains were tested in the feeding inhibition assay there was no relationship between reduction in nematode feeding rate and genomovar. Strains that caused death in the plate-based mortality assay also caused a reduction in consumption rate in the feeding inhibition assay. Large differences in virulence were seen between strains from the same genomovar and strains known to cause severe disease in humans were not consistently the most virulent in either the plate based mortality assay of the feeding inhibition assay. Thus the nematode model may not model mammalian infection caused by the *B. cepacia* complex strains tested.

Strains of *B. pseudomallei* and *B. thailandensis* were also investigated in the plate-based mortality and feeding inhibition assays. The differences in nematode killing by different strains in the plate-based mortality assay reflected differences in virulence as seen in vertebrates. However the results from the feeding inhibition assay did not show the same relationship. Thus the results from the feeding inhibition assay and the plate-based mortality assay do not correlate suggesting feeding inhibition and killing occurs through different mechanisms.

The novel nature of the feeding inhibition assay means that at present no published work on its use with bacterial pathogens exists apart from that reported here (Smith 2002). Jones and Candido (Jones and Candido 1999) utilised a similar assay for the investigation of

feeding inhibition and killing of *C. elegans* in response to chemicals. Through the use of *C. elegans* mutants they showed that conditions that induced a stress response inhibited feeding. The degree of feeding inhibition depended on the amount of stress experienced. The level of stress detected was linked to the concentration of particular chemicals but this varied depending on the compound used. They could not demonstrate any sensory transduction events underlying feeding inhibition and thus could discover no mechanism by which this process occurs. However, Jones and Candido did suggest that feeding inhibition was a survival response in *C. elegans* used to limit damage by the ingestion of toxic compounds encountered in the environment (Jones and Candido 1999). The work reported in this thesis agrees with these results as feeding was also shown to be reproducibly inhibited by sodium salicylate. In addition to chemicals, the data here has shown that the feeding inhibition response may be a defence against bacterial pathogens, as feeding inhibition occurred when *C. elegans* was fed on suspensions of these organisms. Different pathogens were shown to inhibit feeding to a greater or lesser extent. This is presumably in part due to differences in virulence, the initial starting OD_{550nm} of the bacterial suspension and different numbers of nematodes between each experiment. The counting of worms in small aliquots can give an indication of the numbers of worms in larger samples. However the numbers of nematodes in each well of the feeding inhibition assay can only be standardised through the use of specialist worm counting and dispensing machinery (http://www.unionbio.com), which was not available for use in this study. The feeding inhibition assay can only show if one bacterial strain is more virulent than another. It cannot provide information concerning the events that occur to cause feeding inhibition, as it is simply a measure of the change in OD_{550nm} of a bacterial suspension over time. The feeding inhibition assay, unlike the plate-based mortality assay, does not allow the examination of nematodes and thus precludes the observation of particular phenotypic changes in worms, such as aberrant movement and nose-glob phenotypes. Also the

responses in these assays do not always correlate and strains may appear less virulent in the feeding inhibition assay but highly virulent in the plate-based mortality assay and mammalian infection models. Hence, the feeding inhibition assay was inherently difficult to standardise and was not a robust assay of bacterial virulence. The plate-based mortality assay allowed the observation of the events that lead to death in *C. elegans*. It gave results that were consistently reproducible, which mimicked levels of virulence of *B. pseudomallei* in mammals. Therefore, it must be considered the better model of *B. pseudomallei* pathogenicity using *C. elegans* and overall the better tool for the investigation of bacterial virulence.

8.2. Investigation of *B. pseudomallei* pathogenesis using the nematode *C. elegans*.

The interaction of *B. pseudomallei* with the cells of the *C. elegans* intestinal tract was investigated through TEM (Chapter: 5.). After 24 hours post exposure no close association of *B. pseudomallei* with the cells lining the intestine or invasion of these cells was observed. This suggests that intracellular invasion does not occur and that killing of *C. elegans* does not involve the invasion of nematode tissues, intracellular spread and replication of the organism as seen in the mammalian host (Kespichayawattana, Rattanachetkul *et al.* 2000). Thus the interaction of *B. pseudomallei* with the cells of the *C. elegans* gut was not similar to that seen in mammalian tissues. However a number of bacteria appeared to be in the process of cellular division at the point of fixation indicating that these may have been viable (Figure: 5.4). As no invasion of the tissues surrounding the gut was observed but killing still occurs during *B. pseudomallei* infection of *C. elegans*, this suggests that killing may be initially mediated by secreted products. As a saprophytic organism *B. pseudomallei* could be expected to produce a variety of extracellular products

(Haase, Janzen *et al.* 1997; Ellis 1999; Brett and Woods 2000; Dance 2000). This may include the paralysing toxin implicated in the nematode killing process (O' Quinn, Wiegand *et al.* 2001; Gan 2002), other toxins including a putative cell elongating factor and a cytoleathal peptide (Haase, Jensen *et al.* 1997) or proteases (Ellis 1999). These products could damage *C. elegans* cells and eventually degrade the nematode to a level where invasion and digestion of underlying tissues is possible resulting in death of the worm.

TEM allowed the observation of *B. pseudomallei* in the nematode gut early in the infection process. This approach although useful could not provide information concerning the molecular interactions that lead to *B. pseudomallei* virulence in *C. elegans*. To address this, molecular approaches to the investigation of *B. pseudomallei* virulence were explored in chapters: 6 and 7. Firstly *B. pseudomallei* mutants were assessed for their virulence in nematodes (Chapter: 6.). This showed that the *C. elegans-Burkholderia* infection model was capable of identifying mutations that result in a decrease in virulence in both nematodes and mammalian models of infection.

Other workers have also shown that *C. elegans* is a model of disease for a variety of bacterial pathogens including *B. pseudomallei* (Table: 1.1.). O' Quinn *et al* (O' Quinn, Wiegand *et al.* 2001) first described the use of a *C. elegans-Burkholderia* infection model. They showed that *C. elegans* was susceptible to a range of *Burkholderia* strains including *B. cepacia*, *B. thailandensis* and *B. pseudomallei*. Gan *et al* (Gan 2002) then used a similar assay to further investigate *B. pseudomallei* virulence and showed that the differences in virulence of strains seen in nematodes mirrored that seen in mammals as was found in this study. Gan *et al* also used this model to screen a large number of *B. pseudomallei* mutants. From 3,400 transposon mutants, five were isolated that showed reduced virulence in *C.*

elegans. The interrupted genes were not previously known to play a role the in virulence of *B. pseudomalle*i and included a putative amino acid transporter and a putative ABC transporter protein (Gan 2002). To confirm the functional relevance of the interrupted genes to mammalian pathogenesis, each mutant was then screened in BALB/c mice. Mice infected with the mutants displayed an increased time to death. In this study I also identified *B. pseudomallei* mutants attenuated in *C. elegans*; *B. pseudomallei* 6H2 was less virulent in nematodes and mice infected with *B. pseudomallei* 6H2 showed an increased time to death. When the interrupted gene was identified it was found to be a gene not previously known to be involved in the virulence of *B. pseudomallei in vivo*. These data, together with the findings of Gan *et al* (Gan 2002), show that the nematode *C. elegans* can be used to investigate *B. pseudomallei* virulence in the mammalian host.

The success of the *C. elegans-Burkholderia* model in the identification of a transposon mutant with decreased virulence, prompted me to investigate other molecular approaches to the investigation of bacterial virulence factors. The usefulness of *C. elegans* for the screening of a *B. pseudomallei* genomic cosmid library carried in *E. coli*, for clones with increased virulence was explored (Chapter: 7.). However, no single cosmid clone displaying an increased virulence was identified using the feeding inhibition assay. When the levels of *B. pseudomallei* protein expression from cosmids was assessed, no expression was detected using Western blotting, Coomassie Blue staining or silver staining of SDS-PAGE gels. This suggests that the quantities of protein produced were below the detection level of these methods. This then would explain the lack of any effect seen in the *C. elegans* model, as levels may be too low to be biologically efficacious in nematodes. As the feeding inhibition assay relies on the use of a liquid medium, already very small quantities of virulence factors might be further diluted. The plate-based mortality assay exposes worms to high concentrations of the challenge bacterium and its products. Thus,

the use of the plate-based mortality assay, to screen cosmid clones may in retrospect have been more appropriate.

This is the first reported use of an assay based on nematode worms being used for the screening of cosmid libraries for clones with increased virulence. Hence these results cannot be compared to work carried out by other authors using these techniques. However cosmid clones with increased virulence have been identified using other *in vivo* and *in vitro* assay methodologies (Regue 2001; Robey 2002). A further study, using a plate-based assay, may allow the identification of cosmid clones with increased pathogenicity and allow isolation of genes important in nematode killing. As the results presented in thesis have shown, these genes could also be important in the pathogenesis of *B. pseudomallei* in mammalian models of infection.

8.3. Conclusions

The aims of this thesis, stated in section: 1.6. have been met. The investigation of techniques for the study of bacterial pathogenicity using *C. elegans* has lead to the establishment of a nematode model of *B. pseudomallei* infection. When this model was investigated further, assays carried out on solid media were also found to be a better model of *B. pseudomallei* virulence in mammals. *B. pseudomallei* mutants that had a reduced virulence towards nematodes in a plate–based mortality assay also showed a decrease in virulence in mammalian models of infection. Hence *C. elegans* has been found to be a useful model for the study of *B. pseudomallei* pathogenesis. This allows the nematode model described in this thesis to be used as tool for the isolation of attenuated mutants, for further study in mammalian infection models. As the methods used to screen mutants are rapid and cost effective, the study of *B. pseudomallei*-host interactions would be greatly facilitated through the use of nematode models. They may help to reduce animal usage in the initial stages of any study involving the screening of *B. pseudomallei* mutant libraries, while providing valuable information concerning the molecular events that lead to virulence in mammals. Infection models using the *C. elegans* nematode continue to provide valuable information regarding the pathogenicity of a number of other bacterial pathogens (Tan 2002). Thus, the use of the *C. elegans-Burkholderia* model will also continue to provide important information regarding the pathogenesis of *B. pseudomallei*, which may eventually lead to a greater understanding of this relatively poorly characterised pathogen.

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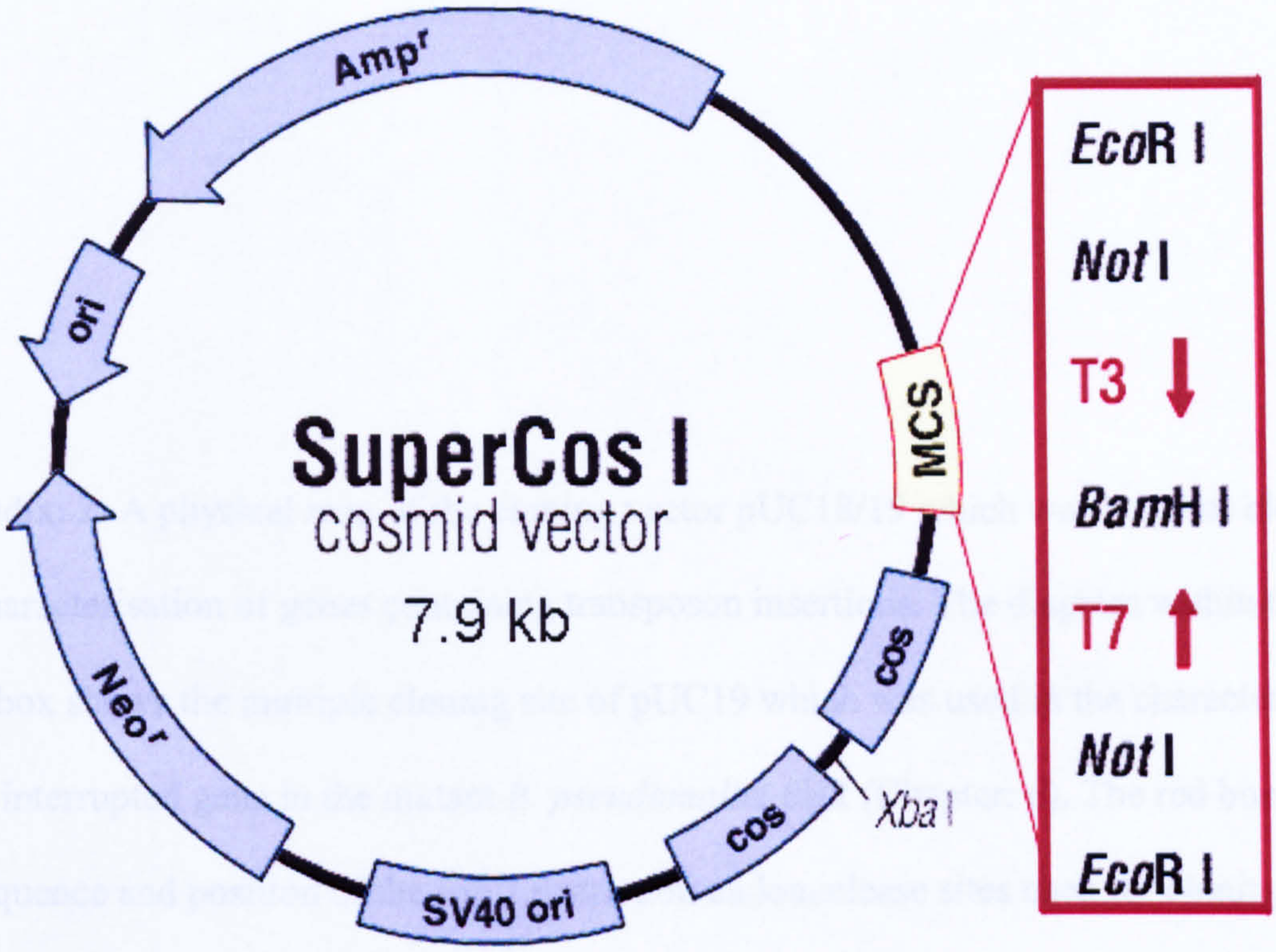
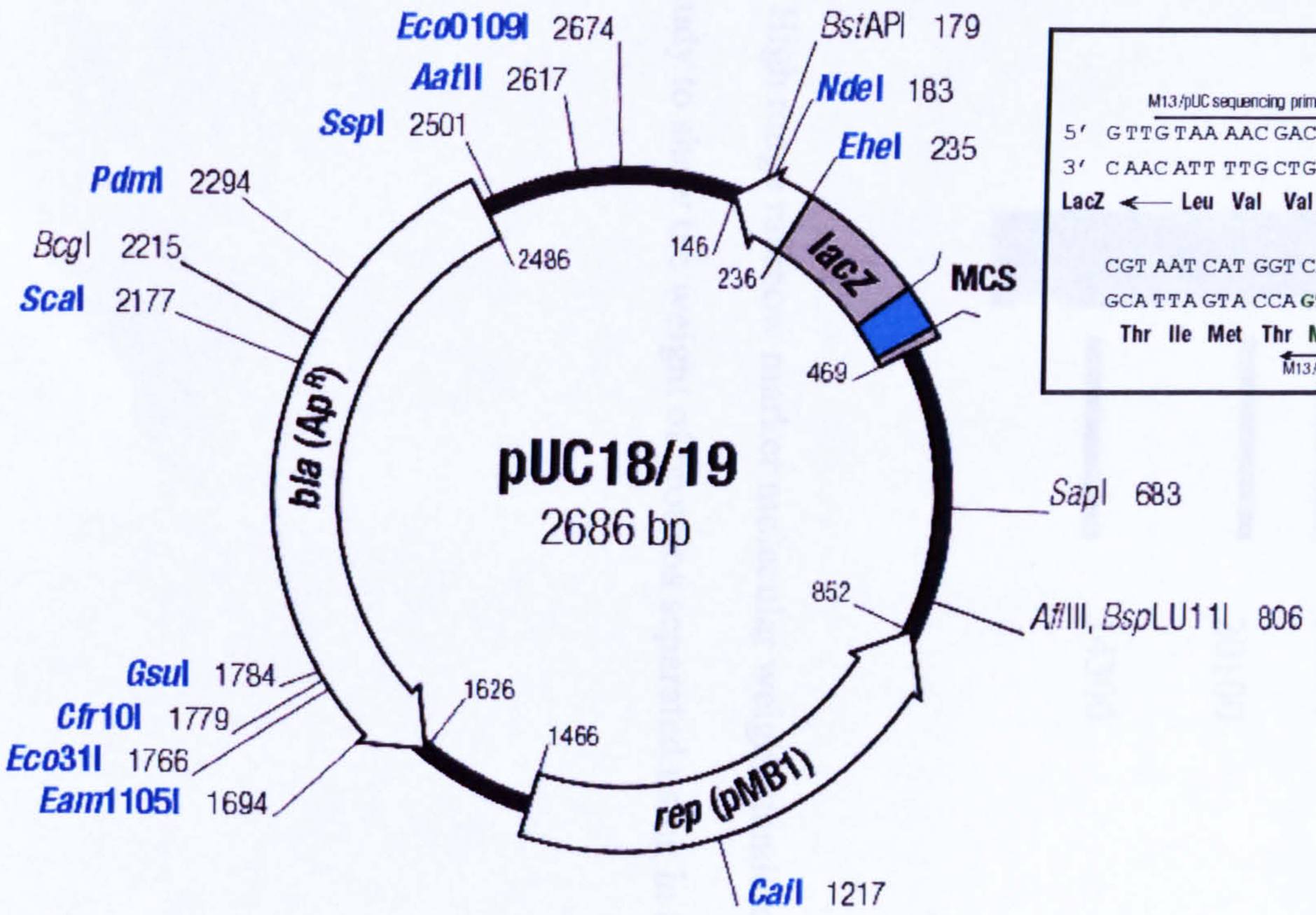
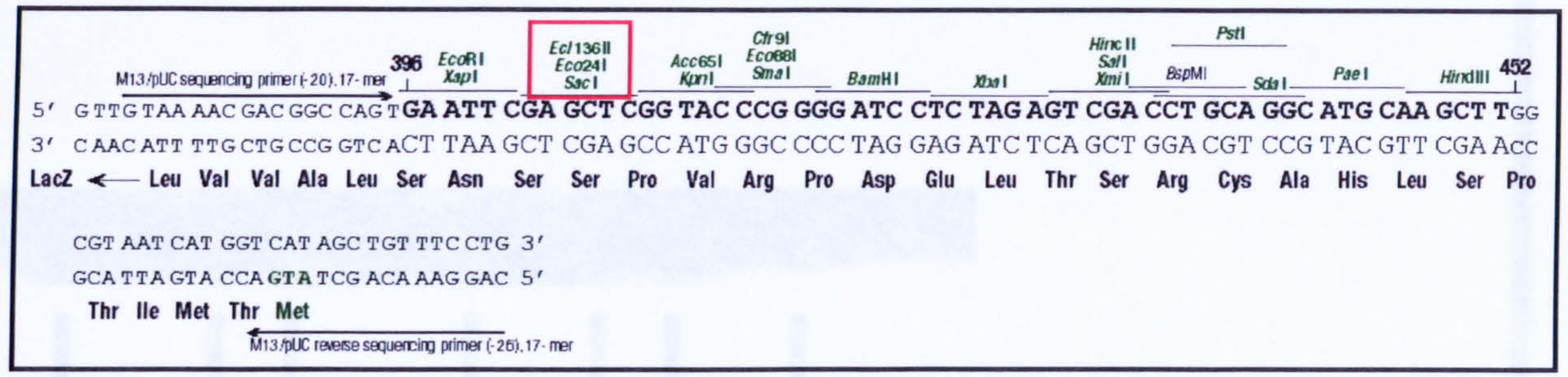
Appendix

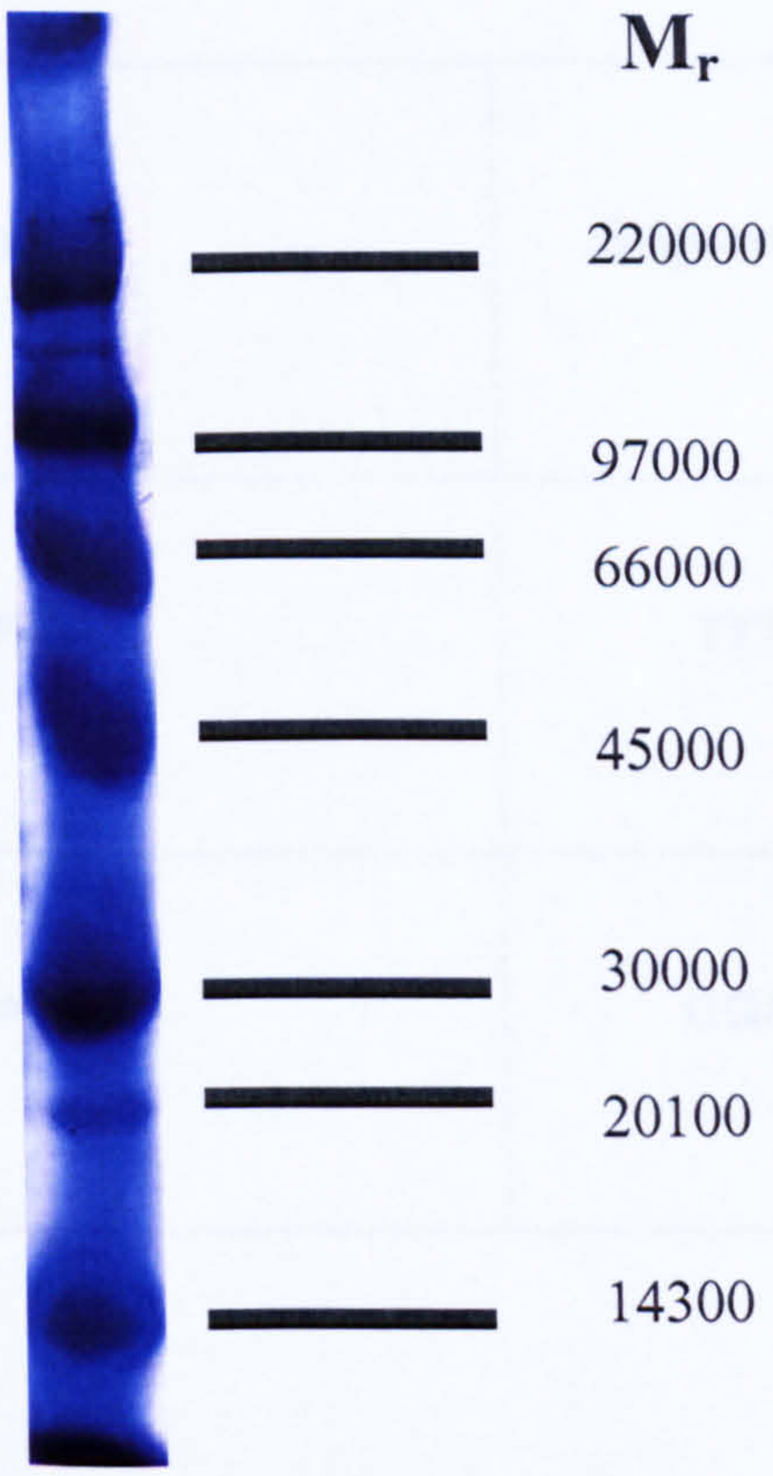


Appendix: 1. Physical map of the supercos-1 cosmid vector. The red box shows the restriction endonucleases that can be used for cloning. Courtesy of Stratagene, UK.

Appendix: 2. A physical map of the cloning vector pUC18/19 which was used for cloning and characterisation of genes containing transposon insertions. The diagram within the black box shows the multiple cloning site of pUC19 which was used in the characterisation of the interrupted gene in the mutant *B. pseudomallei* 6H2 (Chapter: 6). The red box shows the sequence and position of the *sac 1* restriction endonuclease sites used for cloning of *B. pseudomallei* genomic DNA.


Eco0109I 2674
AatII 2617
SspI 2501
PdmI 2294
BcgI 2215
ScaI 2177
BstAPI 179
NdeI 183
EheI 235
2486
146
236
lacZ
MCS
469
bla (Ap^R)
pUC18/19
2686 bp
SapI 683
852
AflIII, BspLU11I 806
GsuI 1784
Cfr10I 1779
Eco31I 1766
Eam1105I 1694
1626
1466
rep (pMB1)
CalI 1217


M13/pUC sequencing primer (-20), 17- mer
396
EcoRI XapI
Ecl136II Eco24I SacI
Acc65I KpnI
Cfr9I Eco88I SmaI
BamHI
XbaI
HincII SalI XmiI
PstI
BspMI
SdaI
PaeI
HindIII
452
5′ GTTGTAAAACGACGGCAGTGA ATT CGA GCT CGG TAC CCG GGG ATC CTC TAG AGT CGA CCT GCA GGC ATG CAA GCT TGG
3′ CAACATTTTGCTGCCGTCACT TAA GCT CGA GCC ATG GGC CCC TAG GAG ATC TCA GCT GGA CGT CCG TAC GTT CGA ACC
LacZ ← Leu Val Val Ala Leu Ser Asn Ser Ser Pro Val Arg Pro Asp Glu Leu Thr Ser Arg Cys Ala His Leu Ser Pro
CGT AAT CAT GGT CAT AGC TGT TTC CTG 3′
GCA TTA GTA CCA GTA TCG ACA AAG GAC 5′
Thr Ile Met Thr Met
M13/pUC reverse sequencing primer (-26), 17- mer



Appendix: 3. High range rainbow marker molecular weight standard (Amersham, UK) used in this study to show the weight of proteins separated using in SDS-PAGE.

Primers	Sequencing primer Sequence (5'-3')
M13 forward	TTT CCC AGT CAC GAC GTT G
M13 reverse	GGA TAA CAA TTT CAC ACA GG

Appendix.4. Primers and primer sequences used in the DNA sequencing of cloned DNA within the multiple cloning site of the plasmid vector pUC19.